

U.S. Application No.
Unknown

International Application No.
PCT/DK99/00258

Attorney Docket No.
PLOUG1.001APC

Date: November 7, 2000

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**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 USC 371**

International Application No.: PCT/DK99/00258
International Filing Date: May 7, 1999
Priority Date Claimed: May 7, 1998 (DK) and May 12, 1998 (US)
Title of Invention: CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT
Applicant(s) for DO/EO/US: Kenn Gerdes, Marie Mikkelsen, Hugo Grønlund, Kim Pedersen,
Peter Kristoffersen

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
3. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
4. (X) A copy of the International Application as filed (35 USC 371(c)(2))
 - a) ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b) (X) has been transmitted by the International Bureau.
 - c) ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
5. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
 - a) ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b) ☐ have been transmitted by the International Bureau.
 - c) ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d) (X) have not been made and will not be made.
6. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.
7. (X) A FIRST preliminary amendment.
8. (X) International Application as published, including:
 - a. (X) Publication Cover Sheet
 - b. (X) 82 pages of disclosure
 - c. (X) Sequence listing in 20 pages
 - d. (X) 22 pages of drawings
 - e. (X) Int'l. Search Report
9. (X) PCT Form PCT/IB/308.

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10. (X) PCT request form.
11. (X) Other Items or information: Indications relating to a deposited microorganism
12. (X) A return prepaid postcard.
13. (X) The following fees are submitted:

				FEES
BASIC FEE				\$1,000
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	99 - 20 =	79 ×	\$18	\$1422
Independent Claims	7 - 3 =	4 ×	\$80	\$320
TOTAL OF ABOVE CALCULATIONS				\$2742
Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28)				\$1371
TOTAL NATIONAL FEE				\$1371
TOTAL FEES ENCLOSED				\$1371

14. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.
15. (X) A check in the amount of \$1371 to cover the above fees is enclosed.
16. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

KNOBBE, MARTENS, OLSON & BEAR, LLP
620 Newport Center Drive
Sixteenth Floor
Newport Beach, CA 92660

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110700

Che A. Chereskin
Signature

Che Swyden Chereskin, Ph.D.
Printed Name

41,466
Registration Number

FILED "OFFICE"

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Gerdes, et al.)	Group Art Unit Unknown
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Appl. No.	:	<u>09/700,130</u>)	
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Filed	:	November 7, 2000)	
)	
For	:	CYTOTOXIN-BASED)	
		BIOLOGICAL)	
		CONTAINMENT)	
)	
Examiner	:	Unknown)	

SUPPLEMENTAL PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination on the merits, please amend the above-referenced application as follows:

IN THE SPECIFICATION:

Please delete the paragraph beginning on page 1, on line 3, immediately after the Title of the Invention and prior to Field of the Invention, and replace it with the following paragraphs:

Cross-Reference to Related Applications

This is the U.S. National Phase under 35 U.S.C. § 371 of International Application PCT/DK99/00258, filed May 7, 1999 which claims priority to Danish Application DK 0627/98, filed May 7, 1998, and U.S. Application 60/085,067, filed May 12, 1998, the disclosures of which are herein incorporated by reference in their entireties.

IN THE SEQUENCE LISTING:

Please replace the originally filed Sequence Listing with the accompanying Substitute Sequence Listing.

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Filed : November 7, 2000

REMARKS

The specification has been amended to correct the filing date of International Application PCT/DK99/00258 to May 7, 1999. Support for this amendment can be found on the copy of the published PCT application submitted on November 7, 2000.

The Sequence Listing has been amended to include the serial number and filing date of the present U.S. Application and the PCT application of which the present application is the U.S. National Phase. The generic term "Bacterium" in Sequences 10 through 43 has been replaced with the genus and species names of the organisms from which these sequences originate. Support for the amendments are found within the Preliminary Amendment filed November 7, 2000 and within the specification as filed on pages 42-45.

CONCLUSION

In view of the foregoing, it is respectfully submitted that the present application is in condition for allowance. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the number below.

The specific changes to the specification and the amended claims are shown on a separate set of pages attached hereto and entitled VERSION WITH MARKINGS TO SHOW CHANGES MADE, which follows the signature page of this Amendment. On this set of pages, the insertions are double underlined while the ~~deletions are stricken through~~.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Nov. 20, 2001

By: Daniel Hart

Daniel Hart
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

Cross-Reference to Related Applications

This is the U.S. National Phase under 35 U.S.C. § 371 of International Application PCT/DK99/00258, filed ~~November 18~~May 7, 1999, which claims priority ~~of~~to Danish ~~application,~~Application DK 0627/98, filed May 7, 1998, and U.S. ~~application~~Application 60/085,067, filed May 12, 1998, the disclosures of which are herein incorporated by reference in their entireties.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Gerdes, et al.)	Group Art Unit Unknown
)	
Int'l Appl. No.	:	PCT/DK99/00258)	I hereby certify that this correspondence and all
)	marked attachments are being deposited with
Int'l Filing)	the United States Postal Service as first-class
Date	:	November 18, 1999)	mail in an envelope addressed to: Assistant
)	Commissioner for Patents, Washington, D.C.
)	20231, on
)	November 7, 2000
)	(Date)
For	:	CYTOTOXIN-BASED)	<i>Che S. Chereskin</i>
		BIOLOGICAL)	Che Swyden Chereskin, Ph.D.,
		CONTAINMENT)	Registration No. 41,466
)	
Examiner	:	Unknown)	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
 Washington, D.C. 20231

Dear Sir:

Preliminary to examination on the merits, please amend the above-captioned U.S. National Phase Application as follows:

IN THE SPECIFICATION

On page 1 of the Specification, on line 3, after the Title of the Invention and before the Field of the Invention statement, please insert --This is the U.S. National Phase under 35 U.S.C. §371 of International Application PCT/DK99/00258, filed November 18, 1999, which claims priority of Danish application, DK 0627/98, filed May 7, 1998 and US application 60/085,067, filed May 12, 1998.--.

On page 71, please cancel the word "CLAIMS" and substitute in its place --WHAT IS CLAIMED IS:--.

IN THE CLAIMS:

Please amend the claims as follows:

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1. (Amended) A method of [conditionally] controlling the survivability of a recombinant microbial cell population, [the method] comprising:

(i) [providing]expressing in the cells of said population a gene coding for a cytotoxic [first kind of] polypeptide[, the gene is selected from the group consisting of: the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally, equivalent polypeptide (said genes collectively being designated herein as) from the *relE* gene family], said gene is expressible in the cells of the population and,] operably linked to [the gene, a regulatable] a regulatory DNA sequence; and

(ii) cultivating the cells [population] under conditions where the [relE]cytotoxic gene [or the gene coding for a functionally equivalent polypeptide] is expressed, wherein the expression [leading to an]results in at least partial killing of the cell population.

2. (Amended) [A]The method according to claim 1, wherein the gene coding for the cytotoxic polypeptide[functionally equivalent polypeptide] from the *relE* gene family is [derived] from a Gram-negative bacterium.

3. (Amended) [A]The method according to claim 2, wherein the [gene coding for the functionally equivalent polypeptide is derived from a] Gram-negative bacterium is selected from the group consisting of: *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.*, *Pseudomonadaceae spp.*, *Helicobacter spp.* and *Synechosystis spp.*

4. (Amended) [A]The method according to claim 1, wherein the gene coding for the cytotoxic polypeptide [functionally equivalent polypeptide] from the *relE* gene family is [derived] from a Gram-positive bacterium.

5. (Amended) [A]The method according to claim 4, wherein the [gene coding for the functionally equivalent polypeptide is derived from a] Gram-positive bacterium is selected from the group consisting of: lactic acid bacterial *spp.*, *Bacillaceae spp.* and *Mycobacterium spp.*

6. (Amended) [A]The method according to claim 5, wherein the [Gram-positive bacterium]Bacillaceae spp. is *Bacillus thuringiensis*.

7. (Amended) [A]The method according to claim 1, wherein the gene coding for the cytotoxic polypeptide [functionally equivalent polypeptide] from the *relE* gene family is [derived] from a species belonging to [Arhae]Archae.

8. (Amended) [A]The method according to claim 1, wherein the gene coding for the cytotoxic polypeptide from the *relE* gene family [gene] is present on the chromosome of the cells.

9. (Amended) [A]The method according to claim 8, wherein the cells [of the population] do not contain a gene coding for a [second type of] polypeptide [that is] capable of counteracting the [cell] toxic effect of the cytotoxic polypeptide from the [RelE] *relE* gene family [polypeptide or the functional equivalent hereof].

10. (Amended) [A]The method according to claim 1, wherein the gene coding for the cytotoxic polypeptide from the [RelE] *relE* gene family[polypeptide or the functional equivalent hereof] is present on an extrachromosomal replicon.

11. (Amended) [A]The method according to claim 1, wherein the regulatory DNA sequence comprises a promoter which regulates the expression of the gene coding for the cytotoxic polypeptide of the [RelE] *relE* [polypeptide or the functionally equivalent polypeptide]gene family at the transcriptional level [by means of a promoter, the function of which is]and wherein said promoter is regulated by the presence or absence of a chemical compound in the cultivation medium.

12. (Amended) [A]The method according to claim 11, wherein [the]said promoter is inducible by [a]said chemical compound.

13. (Amended) [A]The method according to claim 12, wherein [the]said promoter is suppressible by a first [kind of] chemical compound and inducible by a second [kind of] chemical compound [whereby,], and wherein, when the first [kind of] chemical compound is depleted from the medium, the promoter is induced by the second [kind of]chemical compound.

14. (Amended) [A]The method according to claim 1, wherein the cells [of the population comprises]further comprise a gene coding for a second [kind of] polypeptide [that is]wherein said second polypeptide is capable of binding to the [reIE polypeptide or the functional equivalent, said binding resulting in that] cytotoxic polypeptide of the *relE* gene family and inhibiting the toxic effect [of the RelE polypeptide or the functional equivalent is at least partially counteracted].

15. (Amended) [A]The method according to claim 14, wherein the gene coding for the second [kind of] polypeptide is operably linked to a [regulatable] regulatory DNA sequence, [permitting that the gene coding for the second kind of polypeptide]wherein said

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regulatory DNA sequence is suppressed under conditions where the **[gene coding for the RelE polypeptide or the functional equivalent]**cytotoxic polypeptide of the *relE* gene family is expressed.

16. (Amended) [A]The method according to claim 14, wherein the second **[kind of]** polypeptide is from the *relB* gene family **[RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide]**or a homologue thereof.

17. (Amended) [A]The method according to claim 16, wherein the **[functionally equivalent second kind of polypeptide is derived]***relB* gene is from a microbe selected from the group consisting of: a Gram-positive bacterium, a Gram-negative bacterium and an *Archae* spp.

18. (Amended) [A]The method according to claim 1, wherein the expression of the gene from the *relE* gene family **[gene]** is stochastically regulated.

19. (Amended) [A]The method according to claim 18, wherein the **[stochastic]**stochastic regulation is effected by operably linking the gene from the *relE* gene family **[gene]** to a regulatory sequence that comprises an invertible promoter.

20. (Amended) [A]The method according to claim 18, wherein the **[stochastic]**stochastic regulation **[is effected by]**comprises flanking at least part of the regulatory sequence by repeat sequences **[whereby]**wherein the at least part of the regulatory sequence is recombinationally excised.

21. (Amended) [A]The method according to claim 1, wherein the cells **[contain]**comprise a gene coding for a gene product of interest.

22. (Amended) [A]The method according to claim 21 wherein the gene product of interest is an immunologically active gene product.

23. (Amended) [A]The method according to claim 21, wherein the gene product of interest **[is one that is effective in degradation of]**degrades an environmental pollutant.

24. (Amended) [A]The method according to claim 21, wherein the gene product of interest is a pesticidally active product.

25. (Amended) [A]The method according to claim 24, wherein the **[gene coding for the]** pesticidally active gene product is **[derived]** from *Bacillus thuringiensis*.

26. (Amended) A method of confining an extrachromosomal replicon to a microbial cell population, **[the method]** comprising **[the steps of]**:

(i) isolating a microbial cell [**naturally containing**]comprising a gene belonging to the *relE* gene family, wherein said *relE* gene codes for a [**coding for a first kind of**]cytotoxic polypeptide [**that, when it is expressed in the cell, acts as a. toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell**],

(ii) introducing into the cell [**the**]an extrachromosomal replicon [**to be confined, said replicon containing**]comprising a gene coding for a [**second kind of polypeptide that, by binding to the first kind of polypeptide,**]second polypeptide which acts as an antitoxin for said [**first kind of**]cytotoxic polypeptide,

(iii) cultivating the cell under conditions [**where the genes coding for the first and the second kind of polypeptides**]wherein both genes are expressed[, **whereby**]wherein a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the [**first kind of**]cytotoxic polypeptide [**being expressed in the absence of expression of the second kind of polypeptide**].

27. (Amended) [A]The method according to claim 26, wherein the cell [**population consists of cells that**] further comprises a gene coding for a gene product of interest.

28. (Amended) [A]The method according to claim 27, wherein the gene product of interest is selected from the group consisting of: an enzyme, an immunologically active polypeptide, a pesticidally active gene product and a pharmaceutically active gene product.

29. (Amended) [A]The method according to claim 26, wherein [**the**]said extrachromosomal replicon is a plasmid [**occurring in the microbial cells at**]having a copy number which is in the range of 1-30 [**including the range of 1-10 such as the range of 1-5**].

30. (Amended) [A]The method according to claim 26, wherein the microbial cells [**belong to a**]are Gram-negative bacterial species.

31. (Amended) [A]The method according to claim 30, wherein the Gram-negative bacterial species is selected from the group consisting of: *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.* and *Pseudomonadaceae spp.*

32. (Amended) [A]The method according to claim 26, wherein the microbial cells [**belong to a**]are Gram-positive bacterial species.

33. (Amended) A method of post-segregationally stabilizing a plasmid in a microbial host cell population[, **the method comprising the steps of**]comprising:

(i) [**recombinationally inserting into the**]providing a plasmid [(a)]comprising: a gene coding for a [**first kind of**]cytotoxic polypeptide from the *relE* gene family [**selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell**] and [(b)] a gene coding for a [**second kind of**] polypeptide that [(1) **is capable of acting**]acts as an antitoxin for [**first kind of**]said cytotoxic polypeptide [**and (2)**] wherein said antitoxin is capable of being degraded in the host cell at a higher rate than that at which the [**first kind of**] cytotoxic polypeptide is degraded,

(ii) transforming said plasmid into said cells;

(iii) cultivating [**the cell population**]said cells under conditions where the genes coding for [**the first kind and second kind of**]both polypeptides are expressed,

[**whereby**]wherein a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the [**second kind of polypeptide**]antitoxin.

34. (Amended) [A]The method according to claim 33, wherein the gene coding for the [**functionally equivalent**] cytotoxic polypeptide from the *relE* gene family is [**derived**] from a Gram-negative bacterium.

35. (Amended) [A]The method according to claim 34, wherein the [**gene coding for the functionally equivalent polypeptide is derived from a**] Gram-negative bacterium is selected from the group consisting of *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.*, *Pseudomonadaceae spp.*, *Helicobacter spp.* and *Synechocystis spp.*

36. (Amended) [A]The method according to claim 33, wherein the gene coding for the [**functionally equivalent**] cytotoxic polypeptide from the *relE* gene family is [**derived**] from a Gram-positive bacterium.

37. (Amended) [A]The method according to claim 36, wherein the [**gene coding for the functionally equivalent polypeptide is derived from a**] Gram-positive bacterium is selected from the group consisting of lactic acid bacterial *spp.*, *Bacillaceae spp.* and *Mycobacterium spp.*

38. (Amended) [A]The method according to claim 37, wherein the [**Gram-positive bacterium**]*Bacillaceae spp.* is *Bacillus thuringiensis*.

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39. (Amended) [A]The method according to claim 33, wherein the gene coding for the **[functionally equivalent]** cytotoxic polypeptide from the *relE* gene family is **[derived]** from a species belonging to **[Arhae]**Archae.

40. (Amended) [A]The method according to claim 33, wherein the **[second kind of]** polypeptide that acts as an antitoxin is **[selected from the group consisting of the *relE* polypeptide derived from]**a member of the *relB* gene family from **[*E. coli* K-12 and a functionally equivalent polypeptide]**.

41. (Amended) [A]The method according to claim 40, wherein the **[functionally equivalent second kind of polypeptide is derived]**member of the *relB* gene family is selected from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae spp.*

42. (Amended) [A]The method according to claim 33, wherein the plasmid **[contains]**further comprises a gene coding for a gene product of interest.

43. (Amended) [A]The method according to claim 42, wherein the gene product of interest is an immunologically active gene product.

44. (Amended) [A]The method according to claim 42, wherein the gene product of interest **[is one that is effective in degradation of]**degrades an environmental pollutant.

45. (Amended) [A]The method according to claim 42, wherein the gene product of interest is a pesticidally active product.

46. (Amended) [A]The method according to claim 45, wherein the **[gene coding for the]**pesticidally active gene product is **[derived]** from *Bacillus thuringiensis*.

47. (Amended) [A]The method according to claim 33, wherein the microbial host cells **[belong to a]**are Gram-negative bacterial **[species]**cells.

48. (Amended) [A]The method according to claim 47, wherein the **[bacterial species is]**Gram-negative bacteria is selected from the group consisting of *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.* and *Pseudomonadaceae spp.*

49. (Amended) [A]The method according to claim 33, wherein the microbial host cells **[belong to a]**are Gram-positive bacterial **[species]**cells.

50. (Amended) [A]The method according to claim 33, wherein the plasmid **[is one that]** occurs **[in the microbial cells]** at a copy number **[which is]** in the range of 1-30 **[including the range of 1-10 such as the range of 1-5]**.

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51. (Amended) A recombinant cell or cells comprising:
a gene coding for a **[first kind of]** cytotoxic polypeptide **[selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide]** from the *relE* gene family or a variant or derivative thereof, **[said first kind of polypeptide having a toxic effect on the cell, subject to the limitation that]** wherein, when the cell is *E. coli*, the **[gene coding for the first kind of]** cytotoxic polypeptide is not **[derived]** from *E. coli*.

52. (Amended) **[A]** The cell according to claim 51, wherein the cells **[belong to]** are bacterial **[species]** cells selected from the group consisting of *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.* and *Pseudomonadaceae spp.*

53. (Amended) **[A]** The cell according to claim 51, wherein the cells **[belong to a]** are Gram-positive **[bacterial species]**.

54. (Amended) **[A]** The cell according to claim 51, wherein the gene coding for **[*E. coli* K-12 RelE polypeptide or the functional equivalent hereof]** the cytotoxic polypeptide of the *relE* gene family is located on the chromosome.

55. (Amended) **[A]** The cell according to claim 51, wherein the gene coding for the **[*E. coli* K-12 RelE 1 polypeptide or the functional equivalent hereof]** cytotoxic polypeptide of the *relE* gene family is located on an extrachromosomal replicon.

56. (Amended) **[A]** The cell according to claim 51, **[which comprises]** further comprising at least one gene coding for a gene product of interest.

57. (Amended) **[A]** The cell according to claim 56, wherein the gene product of interest is an immunologically active gene product.

58. (Amended) **[A]** The cell according to claim 56, wherein the gene product of interest is one that **[is effective in degradation of]** degrades an environmental pollutant.

59. (Amended) **[A]** The cell according to claim 51, wherein the gene product of interest is a pesticidally active product.

60. (Amended) **[A]** The cell according to claim 51, which comprises a gene coding for a second **[kind of]** polypeptide **[that is capable of binding]** which binds to the **[first kind of]** cytotoxic polypeptide **[whereby]** and reduces the toxic effect **[hereof is at least reduced]**.

61. (Amended) [A]The cell according to claim 51, further comprising, **[operably linked to the gene coding for a first kind of polypeptide, a regulatable]**a regulatory DNA sequence operably linked to the cytotoxic polypeptide.

62. (Amended) [A]The cell according to claim 61, wherein the regulatory DNA sequence **[regulates the expression of the gene coding for the first kind of polypeptide at the transcriptional level by means of]**is a promoter**[, the function of]** which is regulated by the presence or absence of a chemical compound in the cultivation medium.

63. (Amended) [A]The cell according to claim 62, wherein the promoter is inducible by a chemical compound.

64. (Amended) [A]The cell according to claim 62, wherein the promoter is suppressible by a first **[kind of]** chemical compound and inducible by a second **[kind of]** chemical compound **[whereby]**wherein, when the first **[kind of]** compound is depleted from the medium, the promoter is induced by the second **[kind of]** compound.

65. (Amended) [A]The cell according to claim 51, wherein the gene coding for the **[functionally equivalent]**cytotoxic polypeptide is **[derived]** from a Gram-negative bacterium.

66. (Amended) [A]The cell according to claim 65, wherein the **[gene coding for the functionally equivalent polypeptide is derived from a]** Gram-negative bacterium is selected from the group consisting of *Enterobacteriaceae spp.*, *Hemophilus spp.*, *Vibrionaceae spp.*, *Pseudomonadaceae spp.*, *Helicobacter spp.* and *Synechocystis spp.*

67. (Amended) [A]The cell according to claim 51, wherein the gene coding for the **[functionally equivalent]**cytotoxic polypeptide is **[derived]** from a Gram-positive bacterium.

68. (Amended) [A]The cell according to claim 67, wherein the **[gene coding for the functionally equivalent polypeptide is derived from a]** Gram-positive bacterium is selected from the group consisting of lactic acid bacterial *spp.*, *Bacillaceae spp.* and *Mycobacterium spp.*

69. (Amended) [A]The cell according to claim 68, wherein the **[Gram-positive bacterium]***Bacillaceae spp.* is *Bacillus thuringiensis*.

70. (Amended) [A]The cell according to claim 51, wherein the gene coding for the **[functionally equivalent]**cytototoxic polypeptide is **[derived]** from a species belonging to **[Arhae]***Archae*.

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71. (Amended) [A]The cell according to claim 51, which does not contain a gene coding for a second [type of] polypeptide [that is] capable of counteracting the cell toxic effect of the [first kind of polypeptide]cytotoxin.

72. (Amended) [A]The cell according to claim 51, which comprises a gene coding for a second [kind of] polypeptide [that is] capable of binding to the [first kind of polypeptide said binding resulting in that]cytotoxic polypeptide and reducing the toxic effect [of the first kind of polypeptide is at least partially counteracted].

73. (Amended) [A]The cell according to claim 72, wherein the gene coding for the second [kind of] polypeptide is operably linked to a [regulatable] regulatory DNA sequence, [permitting that the gene coding for said second kind of polypeptide]wherein expression is suppressed under conditions where the gene coding for the [RelE polypeptide or the functional equivalent]cytotoxic polypeptide from *relE* is expressed.

74. (Amended) [A]The cell according to claim 72, wherein the gene coding for the second [kind of] polypeptide is a member of the RelB [polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide]gene family.

75. (Amended) [A]The cell according to claim 74, wherein the [functionally equivalent] gene coding for the second [kind of] polypeptide is [derived] from an organism selected from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae spp.*

76. (Amended) [A]The cell according to claim 61, wherein the expression of the gene coding for the [first kind of]cytotoxic polypeptide is stochastically regulated.

77. (Amended) [A]The cell according to claim 76, wherein the [stochastic]stochastic regulation [is effected by]comprises operably linking the gene coding for the first kind of polypeptide to a regulatory sequence that comprises an invertible promoter.

78. (Amended) [A]The cell according to claim 76, wherein the [stochastic]stochastic regulation [is effected by]comprises flanking at least part of the regulatory sequence by repeat sequences [whereby]wherein the at least part of the regulatory sequence is recombinationally excised.

79. (Amended) A composition comprising a cell according to [any of claims 51-78]claim 51.

80. (Amended) A method of limiting the survival of a cell population in a first or a second environment, **[which method comprises]**comprising:

(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide[, **the gene is selected from the group consisting of the gene coding for the E. coli K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and]**from the RelE gene family operably linked to **[the gene,]** a regulatory DNA sequence **[being regulatable by]**wherein an environmental factor **[and which]** regulates the expression of said gene, and

(ii) cultivating the cell population under environmental conditions wherein the gene coding for the cytotoxic polypeptide is expressed, and wherein the expression **[leading]**leads to [an] at least partial killing of the cell population.

81. (Amended) **[A]**The method according to claim 80, wherein the survival of the cell population is limited in a first environment in which the gene is expressed, said cell population thereby being contained in said first environment.

82. (Amended) **[A]**The method according to claim 80, wherein the survival of the cell population is not limited when present in a first environment, **[which]**wherein said first environment could change to a second environment physically and/or chemically distinct from the first environment, **[in which]**and wherein in said first environment the gene **[whose expression results in the formation of]**coding for a cytotoxically active polypeptide is not expressed, **[but]**further, wherein the survival of **[which]**the cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

83. (Amended) **[A]**The method according to claim 80, wherein the survival of a cell population is **[being]** limited by **[providing]**expressing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a **[nucleotide sequence]**second gene encoding an antitoxin repressor substance **[which can]**, wherein said antitoxin repressor substance undergoes a decay when said cells are released to **[the outer]**a different environment **[to an**

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extent whereby]such that the repressor substance is converted to a non-functional form, [whereby]wherein as a result of said decay, the [function of the cells of the population will be gradually limited]cells will die.

84. (Amended) A method of containing an extrachromosomal recombinant replicon to a first [kind of] cell, wherein said replicon is naturally transferable to a second [kind of] cell, [which method comprises] comprising:

providing on the recombinant extrachromosomal replicon a gene [whose expression results in the formation of]coding for a cytotoxic polypeptide from the RelE gene family, [selected from the group consisting of the E. coli K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited]or homologues thereof, said first [kind of] cells [having or being modified to have]comprising a chromosomal replicon comprising a regulatory [nucleotide sequence]gene coding for a [the] gene product [of]which inhibits the expression of said gene coding for a cytotoxic polypeptide or inhibits the [cell function-limiting]cytotoxic effect of the cytotoxic polypeptide and thereby protects said first [kind of] cells, wherein said regulatory gene [being]is lacking in said second [kind of] cell, [whereby,]and if [a cell of] the second [kind]cell receives said extrachromosomal recombinant replicon said cytotoxic gene is expressed and [has a function-limiting effect on said second kind of cell]is toxic to said second cell.

85. (Amended) [A]The method according to claim 84, wherein [the]said regulatory gene product which inhibits [the expression of] the expression of the gene coding for the cytotoxic polypeptide [or the cell function limiting effect of the polypeptide] is selected from the group consisting of: E. coli relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 Pemi polypeptide, the plasmid RP4 ParD polypeptide, and the prophage P1 Phd [polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides].

86. (Amended) A method of stochastically limiting in an environment the survival of a cell population, [the method] comprising:

transforming [the] cells [thereof] with a recombinant replicon [containing]comprising a regulatably expressible gene [which, when expressed in a cell encodes]coding for a cytotoxic polypeptide selected from the [group consisting of the E. coli

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K-12] RelE gene family [polypeptide, the plasmid F CcdB polypeptide, the plasmid R 1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited], wherein the expression of said gene is stochastically induced as a result of recombinational excision of [an excisable] a negatively functioning regulatory nucleotide sequence which[, while present in the cells,] inhibits expression of the gene coding for the cytotoxic polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in [an other] another recombinant replicon present in said cells [of the population containing the replicon].

87. (Amended) [A] The cell according to claim 51, that is an animal cell.

88. (Amended) [A] The cell according to claim 87, that is selected from the group consisting of: a mammal cell, a human cell and an insect cell.

89. (Amended) [A] The method [according to any one of claims 1, 26, 32, 80, 84 or 86] of Claim 1, wherein the [first kind of] cytotoxic polypeptide inhibits translation.

Please add the following Claims:

90. The method according to claim 26, wherein said extrachromosomal replicon is a plasmid having a copy number which is in the range of 1-10.

91. The method according to claim 26, wherein said extrachromosomal replicon is a plasmid having a copy number which is in the range of 1-5.

92. The method according to claim 33, wherein the plasmid occurs at a copy number in the range of 1-10.

93. The method according to claim 33, wherein the plasmid occurs at a copy number in the range of 1-5.

94. The method of claim 80, wherein the cytotoxic polypeptide inhibits translation.

95. The method of claim 84, wherein the cytotoxic polypeptide inhibits translation.

96. The method of claim 86, wherein the cytotoxic polypeptide inhibits translation.

97. The method of claim 80, wherein the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, and the gene coding for the prophage P1 Doc polypeptide.

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98. The method of claim 84, wherein the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, and the gene coding for the prophage P1 Doc polypeptide.

99. The method of claim 86, wherein the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, and the gene coding for the prophage P1 Doc polypeptide.

REMARKS

The Specification and Claims have been amended to comply with the rules of practice in the United States. Support for added Claims 90-99 can be found in the claims as filed. As a result of the amendments herein Claims 1-99 are presented for examination. No new matter has been added herewith.

Conclusion

Should there be any questions concerning this application, the Examiner is invited to contact the undersigned attorney at the telephone number appearing below. Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

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CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

FIELD OF THE INVENTION

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The present invention relates to the field of biologically containing genetically modified microorganisms in a particular environment and vectors to a particular host cell. Specifically, there is provided recombinant vectors and cells containing a proteic killer system based on the *E. coli* RelE polypeptide and functional equivalents of this cytotoxin and method of containing replicons and cells, respectively to particular host cells or particular environments, respectively.

TECHNICAL BACKGROUND AND PRIOR ART

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The increasing application of recombinant DNA technology to engineer novel microorganism which are industrially useful have caused concerns in the general public over the potential risks involved. These concerns are primarily related to the potential harm to humans and to undesirable and/or uncontrollable ecological consequences upon deliberate or unintentional release of such genetically engineered microorganisms (GEMs) into the environment. These concerns have led to the establishment of official guidelines for the safe handling of GEMs in laboratories and production facilities where such organisms are applied. Up till now, such guidelines have primarily been directed to measures of physically containing GEMs in laboratories and production facilities with the aim of reducing the likelihood that workers in such facilities were contaminated, or that the GEMs were to escape from their primary physical environment, such as a fermentation vessel.

It is presently being recognized that the level of safety in the handling of GEMs can be increased by combining physical containment measures with biological containment measures to reduce the possibility of the survival of the genetically engineered organisms if they were to escape from their primary environment.

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Lately, however, concerns have become increasingly focused on potential risks related to deliberate release of GEMs to the outer environment and to the use of GEMs as live vaccines. In this connection there is a strongly felt need to have biological containment systems which subsequent to the environmental release of the GEMs or their administration as vaccines to a human or an animal body, effectively kill the released organisms in a controlled manner or which limit the function of the released GEMs to an extent where such GEMs are placed at a significant competitive disadvantage whereby they will eventually be ousted by the natural microflora of the environment to which they are released.

10

The first systems of biological containment were based on the use of "safe" cloning vectors and debilitated host bacteria. As examples, it has been suggested to select vectors which lack transfer functions or which naturally have a very narrow host range. Examples of debilitated host bacteria are *E. coli* mutants having an obligate requirement for exogenous nutrients not present or present in low concentrations outside the primary environment of the GEMs.

Other suggested biological containment systems have been based on mechanisms whereby the vector is restricted to the GEMs e.g. by using a plasmid vector with a nonsense mutation in a gene, the expression of which is indispensable for plasmid replication or a suppressor mutation in the chromosome, said mutation blocking translational read-through of the message of the gene. A further approach is to maintain the rDNA stably in the host by integrating it into the chromosomes of the GEMs.

Recently, an alternative biological containment strategy has been developed in which a recombinant vector is endowed with a gene encoding a cell killing function which gene is under the control of a promoter only being expressed under certain environmental conditions, such as conditions prevailing in an environment outside the primary environment of the GEMs, or when the vector is unintentionally transferred to a secondary host, or the expression of which is stochastically induced. By using incorporation in a GEM of such a cell killing function and selecting appropriate regulatory sequences, vectors can be constructed which are contained in the primary host cell and/or in a primary physical environment. A cell killing function as defined herein may also be referred to as an active biological containment factor.

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If a stochastically induced mechanism of expression regulation is selected for such a biological containment system, a population of GEMs containing the system will, upon release to the outer environment or if used as a live vaccine, be subjected to a random cell killing which will lead to an increase of the doubling time of the host cell population or eventually to the disappearance of the organisms.

The above-mentioned genes encoding cell killing functions are also frequently referred to as "suicide" genes, and biological containment systems based upon the use of such genes, the expression of which are regulated as defined above, are commonly described as conditional lethal systems or "suicide" systems. Up till now, several such cell killing functions have been found in bacterial chromosomes and in prokaryotic plasmids. Examples of chromosomal genes having cell killing functions are the *gef* (Poulsen et al., 1990) and *relF* (Bech et al., 1985) genes from *E. coli* K-12. Examples of plasmid encoded suicide genes are *hok* and *flmA* (Gerdes et al., 1986) genes isolated from plasmids R1 and F, respectively, the *snrB* gene also isolated from plasmid F (Akimoto et al., 1986) and the *pnd* gene isolated from plasmids R16 and R483 (Sakikawa et al., 1989 and Ono et al., 1987). Common features of these genes are that they are transcribed constitutively, regulated at a post-transcriptional level, and that they all encode small toxic proteins of about 50 amino acids and that their translation is controlled by antisense RNA. The application of the *hok* gene in a biological containment system has been disclosed in WO 87/05932.

An alternative biological containment system is disclosed in WO 95/10614 which is based on the use of genes, the expression of which in a cell where the gene is inserted, results in the formation of mature forms of exoenzymes which are hydrolytically active in the cytoplasm of the cell and which can not be transported over the cell membrane. When such enzymes are expressed, the normal function of the cell becomes limited to an extent whereby the competitiveness, and hence the survival, of a population of such cells is reduced.

The stable maintenance of low copy-number plasmids in bacteria is secured by a number of plasmid-borne gene systems, one of which is based on killing of plasmid-free cells (also termed post-segregational killing). This regulated killing is based on a toxin-antidote principle, i.e. a two-component system comprising a stable toxin and an un-

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stable antidote for the toxin. One such system, which is referred to as a proteic killer gene system is based on protein toxins and protein antidotes (reviewed by Jensen and Gerdes, 1995). The natural function of such systems is to provide stable maintenance of plasmids and it has not been suggested previously to utilize the systems as the basis for confining GEMs to a particular environment.

The *E. coli relB* operon encodes three genes, *relB*, *relE* and *relF* (Bech et al., 1985). It has now been found that *relE* encodes a cytotoxin whose overproduction is lethal to host cells and that the *relB* gene encodes an antitoxin that prevents the lethal action of RelE. When present on a plasmid, the *relBE* operon was able to stabilize the inheritance of a mini-R1 test plasmid. It was also found that *relBE* homologous gene systems are found in a wide variety of Gram-negative and Gram-positive bacteria and in *Archae*.

These results show that the *relBE* genes constitute a new ubiquitously occurring family of gene systems that belongs to the proteic plasmid stabilization systems.

These findings has opened up for an alternative, highly effective and versatile biological containment system as it is described in the following. Importantly, it has been discovered that such a system involves the significant advantage that the frequency of spontaneously occurring mutants of microorganisms that have become resistant to the lethal effect of these cytotoxins is very low. This implies that this biological containment system is very safe.

SUMMARY OF THE INVENTION

Accordingly, the invention pertains in a first aspect to a method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene,

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a regulatable regulatory DNA sequence and (ii) cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

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In a further aspect there is provided a method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

(i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,

(ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,

(iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In a still further aspect, the invention relates to a method of post-segregationally stabilizing a plasmid in a microbial host cell population, the method comprising the steps of

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(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

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(ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed
5 as a result of the faster degradation of the second kind of polypeptide.

In yet other aspects, the invention provides a recombinant microbial cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, a gene coding for a functionally equivalent polypeptide

10 hereof or a variant or derivative of any such polypeptide, said first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*, and a composition comprising such cells.

15 The invention also pertains to several methods of containing cells or replicons including

(1) a method of limiting the survival of a cell population in a first or a second environment, which method comprises

20

(i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4

25 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

30

(ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population,

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(2) a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell, and

(3) a method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon containing a regulatably expressible gene which, when expressed in a cell, encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of re-combinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

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DETAILED DISCLOSURE OF THE INVENTION

One objective of the present invention is to provide a novel approach to conditionally controlling the survivability of a recombinant microbial cell population. This approach
 5 is based on the use of what is generally referred to as proteic killer systems which have been reviewed i.a. by Jensen et al., 1995. These systems consist of two components, a cytotoxin polypeptide (also referred to herein as a first kind of polypeptide) and a corresponding antitoxin or antidote polypeptide (also referred to herein as a second kind of polypeptide) that by binding to the cytotoxic polypeptide inhibits the toxic
 10 effect hereof. A general characteristic of such proteic killer systems is that the antitoxin component in contrast to the toxin component is susceptible to protease degradation, resulting in a decay of the antitoxin polypeptide.

As used herein, the expression "microbial cell" includes any prokaryotic and eukaryotic
 15 cells as well as cells of *Archae* species. Thus this expression includes cells of bacterial species, fungal species, animal species including invertebrates, vertebrates, mammals, humans and insects, and plant cells.

Thus, in one aspect of the invention there is provided such a method of conditionally
 20 controlling the survivability of a recombinant microbial cell population that comprises as the first step, providing in the cells a gene coding for a cytotoxic first kind of polypeptide, which gene is selected from the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a structurally and functionally equivalent polypeptide and, operably linked to the gene, a regulatable regulatory DNA sequence.

25 Genes which are structurally and functionally equivalent to *relE* are herein collectively referred to as the *relE* gene family or as *relE* homologues. This group of genes including the *E. coli* plasmid P307 derived *relE* homologue encompasses genes the gene products of which have cytotoxic effects and which, relative to *E. coli* K-12 RelE,
 30 have at least 20% such as at least 30% e.g. at least 40% identical and conserved amino acids. The sequences listed in the below Table 1.5 are putative RelE homologues.

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Whereas, in accordance with the invention, presently preferred recombinant microbial cells are prokaryotic cells such as Gram-negative and Gram-positive bacterial cells, it has been found that the survivability of other microbial cells such as *Archae*, yeast cells, fungal cells, animal cells including human cells and plant cells and replicons of
5 such organisms can be conditionally controlled using the methods of the present invention.

In the present context, the expression "conditionally controlling" refers to a construction of the microbial cell which permits that the gene coding for the cytotoxic polypep-
10 tide can be expressed under certain pre-determined environmental conditions whereas under other such conditions, the gene is not expressed. Hence, the survivability of the microbial cells can be made dependent on certain pre-selected conditions.

In accordance with the invention, the survivability of microbial cells is controlled by
15 the expression in the cells of a cytotoxic polypeptide selected from *E. coli* K-12 RelE polypeptide and a functionally equivalent polypeptide. As used herein, the term "cytotoxic" refers not only to a loss of the ability of microbial cells containing the toxin-encoding gene to remain viable as determined by the capability to propagate in media which, under identical environmental conditions, support unrestricted growth of similar
20 cells not containing the toxin-encoding gene, but also to cells having, as a result of the expression of the polypeptide-encoding gene, a limited cell function, the latter expression denoting that the growth of a cell as manifested i.a. by the synthesis of new cell material and the rate of replication of the cell is decreased.

25 During the experimentation leading to the invention it was surprisingly found that a range of cytotoxic polypeptides according to the invention have the effect that they inhibit translation of genes. This general effect of RelE polypeptides and functionally equivalent polypeptides appears to represent a hitherto undiscovered mechanism for controlling survivability of cells and thus for containment of such cells or replicons in
30 accordance with the methods of the present invention.

Whereas the recognizable manifestation of such limited cell function may ultimately be cell death, it may also be a reduced cell growth appearing as a reduced rate of replication resulting in a reduced increase of cell numbers within a certain period of time as a

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result of an increase of the lag phase and/or of the cell doubling time. Other manifestations may be a relatively increased requirement for one or more nutrient components or a relatively higher susceptibility to detrimental environmental factors such as sub-optimal temperatures or cell damages caused by toxic substances.

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In the present context, the expression "a functionally equivalent polypeptide" refers to a polypeptide that has substantially the same effect on the survivability of microbial cells as the RelE polypeptide of *E. coli* K-12. As it is shown herein, a variety of Gram-positive and Gram-negative bacteria and *Archae* organisms comprise DNA sequences

10 showing homology with the RelE polypeptide. To the extent gene products of structural homologues of the *relE* gene product show an effect on microbial cell survivability as it is defined above, they are encompassed by the present invention. It will also be appreciated that the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene
15 product of which has retained at least part of the cytotoxic function of the gene product of the non-modified sequence.

In the above method, a regulatable regulatory DNA sequence is operably linked to the
20 gene coding for the cytotoxic polypeptide. In accordance with the invention, such a regulatory sequence can be one with which the gene coding for the cytotoxic polypeptide is naturally associated or it can be a sequence with which the gene is not naturally associated. In the present context, the term "regulatory DNA sequence" is intended to indicate a DNA sequence which directly or indirectly regulates the expression
25 of the gene coding for the cytotoxic polypeptide at the level of transcription or at the level of translation or at the level of protein function. The regulatory DNA sequence may thus be one, the function of which results in a suppression or inhibition of the activity of the regulatable promoter.

30 Such regulatory DNA sequences are referred to herein as "negatively functioning regulatory DNA sequences". One interesting example of such a regulatory DNA sequence is a sequence coding for a repressor substance which represses the expression of the gene coding for cytotoxically active polypeptide and which substance may, when a cell containing it is released to a human or an animal body or to the outer envi-

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ronment where the substance is no longer being expressed, undergo a decay whereby the repression of expression of the cytotoxin-encoding gene is gradually reduced and eventually, when the decay of the repressor is completed, the repression is removed.

- 5 Another example of such a regulatory DNA sequence is a sequence encoding a polypeptide that acts as an antidote or antitoxin for the cytotoxic polypeptide. Such a sequence include the *relB* gene derived from the *relBE* operon of *E. coli* K-12 which is capable of binding to the RelE polypeptide and thereby inhibiting its effect. As also shown herein, sequences encoding such antitoxins can be found in Gram-negative and
- 10 Gram-positive bacteria and in *Archae*. Such homologues of the *relB* sequence are encompassed by the present invention.

- In preferred embodiments of the invention, the regulatory DNA sequence may be present in the cell in one or more recombinant replicons and it may be contained in the
- 15 same replicon as that containing the cytotoxin-encoding gene or in a different recombinant replicon.

- One way whereby the expression of the cell function-limiting cytotoxic polypeptide can be regulated is by providing in the cell a gene coding for the polypeptide, which
- 20 gene is regulated at the level of transcription. The regulation at the level of transcription may be carried out in various ways including a regulation by means of a promoter, regulated by one or more factors. These factors may either be ones which by their presence ensure expression of the gene coding for polypeptide or may, alternatively, be factors which suppress the expression of the gene so that their absence causes the
- 25 polypeptide to be expressed.

- Factors regulating the activity of the promoter as defined above may be selected from a variety of factors. Thus, the expression of the gene encoding the polypeptide may be determined by the environmental conditions, by the physiological state of the cells,
- 30 or by a cyclical or stochastic event. In the present context, the term "cyclical event" is understood to mean a cyclically recurrent event causing changes in certain factors known to be potentially useful in affecting the expression of genes such as temperature conditions, changes in light intensity or hormonal changes. The term "physiologi-

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cal state of the cells" denotes factors such as cell density or the growth phase of cells.

In accordance with the invention, advantageous promoter regulating factors are readily
5 regulatable factors including the presence or absence of a certain chemical substance
in the environment or the physical conditions in the environment such as the prevailing
temperature or other physical factors (e.g. the intensity of the light in the environ-
ment). Thus, it is possible to envisage containment systems as presently claimed, in
which the gene coding for the cytotoxic polypeptide is expressed when a certain
10 chemical substance present in a first environment such as the fermentation medium in
which the cell is propagated, is not present in a second environment to which the cell
is released, or when a factor required for the growth or survival of the cell is no longer
present, or the factor is one which, when it is depleted or exhausted from an envi-
ronment of the cell, has the desired effect, viz. that the gene is expressed.

15 The promoter regulating the transcription of the gene coding for the cytotoxic poly-
peptide can also become activated in a second environment of the cell by a chemical
substance which is not present in a first environment of the cell, but which is present
in the second environment in sufficient quantities to activate the promoter. Similarly,
20 the promoter may be a promoter which is activated by a shift in temperature, such as
a shift from a higher temperature in a first environment as e.g. a fermentation vessel,
to a lower temperature prevailing in an outside second environment, or the intensity of
light, in that the promoter may be one which is activated in the presence of light of
sufficient intensity, but is inactive in the darkness prevailing in a first environment
25 such as a fermentation vessel.

Where microbial cells as defined herein are cells that are to be released to the outer
environment in a controlled manner, e.g. to a restricted area of land or to the intestinal
tract of a human or an animal, the regulatable promoter may be one which is regulated
30 chemically, i.e. by the presence or absence of a certain chemical substance in the en-
vironment of the cells as it has been explained above.

However, the regulatable promoter is advantageously a promoter which is activated
cyclically, e.g. by changes of the temperature, or by a stochastic event. The term

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"stochastic event" as used herein is intended to denote an event which occurs at random at a certain frequency per cell per generation or frequency per unit time which, in accordance with the invention, may result in a limitation of the function of the cells in which the activation of expression of the cytotoxic polypeptide occurs, optionally to an extent which leads to the death of the cells. The stochastic event may be occasioned by periodic inversions of the region carrying the promoter or by recombinational excision of a recombinationally excisable negatively functioning regulatory DNA sequence as defined above.

10 It should be noted that in order to ensure a general applicability of the present invention, the promoter used to initiate transcription of the gene coding for the toxic polypeptide is preferably a promoter which is capable of causing expression of said gene in a wide range of cells.

15 In case of regulatable transcription of the polypeptide, the regulatory DNA sequence may e.g. be a promoter isolated from bacterial operons involved in the biosynthesis of amino acids or from bacterial genes, the transcription of which is activated late in the stationary growth phase or from bacterial genes involved in the synthesis of cell surface structures such as fimbriae. Examples of suitable promoters include the *E. coli trp* promoter which becomes activated in the absence of tryptophan, the bacteriophage λ P_R and P_L promoters controlled by temperature sensitive regulatory DNA sequences, the *Bacillus subtilis* sporulation gene promoters which are activated during sporulation, and the *E. coli* and *Salmonella* fimbriae gene promoters which are activated stochastically.

25 In case of chemically regulatable promoters, the chemical substance, the presence or absence of which determines the activation of the promoter, is suitably selected from carbon or nitrogen sources, metabolites, amino acids, nucleosides, purine or pyrimidine bases or metal ions. When the chemical substance is one which, when present,

30 suppresses promoter activity, it is preferably a substance which rarely occurs in the natural environment in such concentrations that the promoter would not be activated when the cell is released to the natural environment. One example of such a promoter is the *trp* promoter which is repressed in the presence of tryptophan in the environment of the cell, but which is derepressed in the absence of sufficient amounts of

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tryptophan in the environment. A containment system according to the invention using the *trp* promoter or another promoter being regulated in the same manner, might therefore comprise an amount of tryptophan in a first environment, such as a fermentation vessel, which is sufficient to repress the promoter in such an environment, the promoter, however, being derepressed when the cell is released from the first environment to a second environment, e.g. the outer environment which usually contains very low amounts of tryptophan or no tryptophan at all.

It is also possible to select a promoter that is regulated by the absence or presence of one or more compounds in exudates of plants colonized with a recombinant organism according to invention.

In this context, another useful promoter is an arabinose inducible promoter including that contained in the plasmid pBAD (Guzman et al., 1995). Without arabinose added to the growth medium, the pBAD promoter is completely turned off. However, in the presence of arabinose, strong transcription is induced. This particular promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off. The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose, the promoter is not induced. However, if cell growth depletes the medium for glucose, then the promoter will be induced. Therefore, such a plasmid is suitable for the conditional turning on and off the expression of gene, in particular toxin-encoding genes as described herein.

Accordingly, in one embodiment of the invention the method is used to contain microbial cells wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

Another example of a regulatable promoter, the activation of which is determined by a chemical substance is the *lac* promoter which is inducible by e.g. isopropyl- β -D-thiogalactopyranoside (IPTG).

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As mentioned above, the regulatable promoter may be a promoter, the activity of which is determined by the temperature prevailing in the environment of a cell containing the gene coding for the cell function-limiting cytotoxin and a regulatable promoter regulating the expression of the gene. In such a case, the regulation of the promoter is advantageously obtained by the presence in the cell of a gene coding for a temperature sensitive repressor for the promoter. As one typical example, the λ promoters including those mentioned above may be regulated by a temperature sensitive λ cl repressor.

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Promoters which are activated stochastically by periodic inversions of the promoter region (in the present context, such promoters are also termed as "invertible promoters" and "inversional switch promoters") and which are useful for the purposes of the present invention include as examples the *hin*, *cin* and *gin* promoters. One particularly useful invertible promoter is the *fimA* promoter which is one *E. coli* fimbriae promoter. The activation (inversional switch) of this promoter is regulated by the gene products of the two genes which for the present purposes is termed the "on" and the "off" genes, the on gene product inducing a switch from off (inactive) to on (active), and the off gene product inducing a switch from on to off. In a wild-type *E. coli* cell where the *fimA* gene and its associated promoter is present in one copy on the chromosome, the inversional switch occurs with a switching frequency of about one cell/1000 cells/generation. It is, however, possible to regulate the frequency of the inversional switch as required by regulating the dosage of expression of the *on* and *off* genes. This is e.g. effected by means of suitable promoters transcribing into the on and off genes. The frequency of transcription initiation by these promoters will then determine the relative dosage levels of the *on* and *off* gene products being formed.

In accordance with the invention, one particular method of stochastically regulating the expression of the gene coding for the toxic polypeptide is the induction of the gene expression as a result of recombinational excision of an excisable negatively functioning regulatory DNA sequence which, while present in the cell, inhibits expression of the gene. In the present context, the term "recombinational excision" refers to the result of a naturally occurring phenomenon of genetic recombination (cross-over) whereby DNA sequences in replicons, in a controlled process, pair, break and rejoin to

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form recombinant replicons by the sequential action of enzymes acting on the DNA. The frequency of recombinational events in a cell depends i.a. on the degree of homology between paired complementary nucleotide sequences and on the length of the complementary sequences. Thus, it has been shown that about 50 base pairs of homology may be required to obtain recombination in a bacterial cell.

When a negatively regulatory DNA sequence is inserted between directly repeated nucleotide sequences of a sufficient length in a recombinationally proficient cell which, in accordance with the invention contains a gene coding for the toxic polypeptide, recombination between the repeats results in the recombinational excision of the negatively regulatory DNA sequence permitting the gene to be expressed.

Accordingly, the phenomenon of recombinational excision implies that a DNA subsequence, i.e. the negatively regulatory DNA sequence, is excised from a longer DNA sequence through a recombination event. In essence, the longer DNA sequence is cleaved on either side of the subsequence and the fresh ends are joined, leaving out the subsequence. Recombination occurs between sufficient homologous flanking nucleotide subsequences. Thus, with DNA of the general structure W-X-Y-X-Z, X being a repeated sequence and Y being a negatively regulatory DNA sequence, this could combine to form W-X-Z, with the Y subsequence being excised.

As mentioned above, the frequency of the recombination can be determined by varying the lengths of the repeats and/or the distance between the repeats. Furthermore, the frequency may be varied by using repeat sequences of varying homologies. Thus, nucleotide sequence repeats being 100% homologous and having a size which does not impair recombination will result in a high recombination frequency and hence, in a high frequency of recombinational excision of the negatively regulatory sequence, whereas mismatches within complementary sequences will reduce the recombination frequency depending on the degree of mismatch. As an example, it has been found that 10% divergence between nucleotide sequence repeats may reduce the recombination frequency 40-fold.

Accordingly, the microbial cell comprising the gene coding for the cytotoxic polypeptide may, in accordance with the invention, be a cell containing a regulatory DNA se-

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quence which is a recombinationally excisable negatively functioning regulatory DNA sequence being flanked by a first flanking nucleotide sequence and a second flanking nucleotide sequence substantially homologous with the first flanking sequence. As used herein, the term "substantially homologous with" is used to indicate that the degree of homology is sufficient to result in a desired frequency of recombination. In certain embodiments it may, in order to obtain a desirable maximum frequency of recombination, be advantageous to use direct repeats, i.e. sequences being 100% homologous, whereas in other embodiments where a moderate degree of cell function limitation is desirable, it is appropriate to use repeats which are more or less heterologous, but still allowing a desirable lower frequency of recombination to occur. Accordingly, in the present context, the term "sufficiently homologous" is used to indicate a degree of homology between two flanking nucleotide sequence repeats which results in a desired frequency of recombinational events in a cell containing the gene coding for the toxin polypeptide and a negatively regulatory DNA sequence.

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As it also has been mentioned above, the frequency of recombination depends on the lengths of the flanking sequences. In useful embodiments of the invention, flanking sequences are used which have a length being in the range of 100-5,000 base pairs. In certain preferred embodiments, it is advantageous to use flanking sequences, the length of which is in the range of 200-3,000 base pairs. As the flanking sequences can be used any nucleotide repeats of sufficient lengths and homology as it has been defined above. As one useful example of flanking sequences may be mentioned the chloramphenicol resistance gene having a size of about 900 base pairs and which occurs in the plasmid pBR325. Another example of a useful nucleotide sequence which, when inserted as repeats, results in recombination, is a subsequence of the *rrnB* gene isolated from the plasmid pKK3535 (Brosius et al., 1981, Plasmid, 6:112-118) having a size e.g. in the range of 500 to about 3,000 base pairs, such as 598 base pairs.

25

In one interesting embodiment of the invention, the excisable negatively regulatory DNA sequence operably linked to the gene encoding the cytotoxic polypeptide is a gene encoding an antisense RNA which forms an RNA-RNA duplex with said the messenger RNA of the polypeptide-encoding gene and thereby, when it is expressed, inhibits translation of said gene coding for the polypeptide.

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In another useful embodiment of the present invention, the recombinationally excisable negatively regulatory DNA sequence is a gene encoding a polypeptide repressor of transcription of the polypeptide-encoding gene. Such a repressor may, e.g. be a *lac* repressor including the repressor encoded by the *LacIq* gene. It will be appreciated

5 that the negatively regulatory DNA sequence can also be a gene coding for RelB anti-toxin or functionally equivalents hereof.

In a further useful embodiment of the invention, the excisable negatively regulatory DNA sequence is a transcription termination sequence, preventing the transcription of
10 the cytotoxic polypeptide-encoding gene. In one specific embodiment of the invention, such a suitable terminator sequence is the *rpoCt'* transcription terminator isolated from the plasmid pHBA102rpoCt (Squires et al., 1981, Nucleic Acid Res., 9:6827-6839).

Negatively regulatory DNA sequences which, in accordance with the invention, are
15 suitable, can be isolated from DNA sequences derived from a virus, or a prokaryotic or eucaryotic cell. Thus, sources of the DNA sequence include bacterial chromosomes, bacterial plasmids, prokaryotic viruses, eucaryotic viruses, eucaryotic plasmids, or eucaryotic chromosomes.

20 In preferred embodiments of the invention, the excisable negatively regulatory DNA sequence and the first and second flanking sequences, both as defined above, is provided in the form of a "cassette" which term is used herein to describe a readily insertable DNA sequence comprising at least the above-mentioned sequences and optionally the gene coding for the cytotoxically active polypeptide, and optionally further
25 nucleotide sequences including as examples a suitable marker such as a gene coding for antibiotic resistance. In the present context, the term "insertable" denotes that the cassette as defined herein is provided with suitable restriction sites at both ends allowing for insertion in a replicon having the same restriction sites. Accordingly, such preferred restriction sites include sites which occur frequently in replicons where inser-
30 tion is desirable or alternatively, restriction sites which may be easily provided in such replicons.

It will be understood that, in accordance with the invention, a cassette as defined above and which does not comprise the gene coding for toxin polypeptide and opera-

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bly linked to the negatively regulatory DNA sequence, may be inserted in a replicon which is different from the replicon containing said gene. Optionally, the cassette as defined above is inserted in a first replicon such as e.g. a transposon and subsequently inserted via the transposon into the chromosome to obtain a cell as defined herein.

5

As it has been explained above, the activation of certain invertible promoters such as the *fimA* promoter or functional homologues hereof is regulated by the gene products of an *on* gene and an *off* gene. It will be understood that this mechanism of promoter regulation provides the possibility of using the *off* gene or a functional homologue

10 hereof as a negatively regulatory DNA sequence which may be inserted in the microbial cell as defined herein, as a recombinationally excisable DNA sequence in the manner explained in details above. Accordingly, in one embodiment, the present invention provides a microbial cell wherein the toxin-encoding gene is stochastically expressed as a result of recombinational inversion of an invertible promoter sequence.

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In plasmids, inherent mechanisms occur whereby multimer resolution of the plasmid during replication takes place. As exemplified by the broad host range plasmid RP4, this resolution system may comprise (1) a gene coding for a multimer resolving enzyme, a resolvase and (2) a site for the site-specific resolvase-mediated resolution. In
20 plasmid RP4 the gene coding for the resolvase is *parA* and the site for the resolution is designated *mrs*. If two *mrs* sites are placed in direct orientation, a DNA sequence inserted between those two sites may, if the *parA* gene is present in the same host cell, be deleted at a relatively high frequency whereby a site-specific recombination system is provided. In useful embodiments the *parA* gene may be located in trans.

25

It has been found that such a site-specific recombination system provides a useful mechanism for stochastically regulating the expression of a gene such as the gene coding for the toxic polypeptide as defined herein, since the site-specific recombination may be used to obtain recombinational excision of a negatively regulatory DNA

30 sequence as defined above.

Accordingly, in one interesting embodiment, the present invention provides a microbial cell as defined herein in which the negatively regulatory DNA sequence is a sequence flanked by a first site for a site-specific resolution recombinase and a second site for

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site-specific resolution, the second site being recognizable by the same or a functionally equivalent multimer resolving enzyme as is the first site, whereby the regulatory sequence is recombinationally excisable in the cell. In a specific embodiment, the gene coding for the multimer resolving enzyme is located *in trans* relative to the sites for site-specific resolution. In the present context, one useful example of a suitable gene is the *parA* gene isolated from plasmid RP4.

In accordance with the invention, the method of controlling the survivability of microbial cells can be based on providing in the cells a gene coding for a cytotoxic polypeptide that is structurally and functionally equivalent to the *E. coli* RelE polypeptide (the *relE* gene family). Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*, *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori* and *Synechosystis* spp, the latter organisms belonging to the group of cyanobacteria. The gene may also be derived from the chromosome and other replicons of Gram-positive bacteria including lactic acid bacteria such as *Streptococcus* spp including *Streptococcus pneumoniae*, *Bacillaceae* spp. such as *B. thuringiensis*, and *Mycobacterium* spp. and from species belonging to *Arhae* such as *Methanococcus jannaschii* and *A. fulgidus*. Such genes include those that are defined herein as belonging to the *relE* gene family. The RelE equivalent polypeptide from *M. jannaschii* was shown to be toxic for *E. coli* when expressed in this organism.

However, genes coding for cytotoxins of other proteic killer systems and which are therefore functional equivalents of the *E. coli* K-12 RelE polypeptide can also be used in accordance with the invention for conditionally controlling the survivability of microbial cells. Such genes include the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide and the gene coding for the prophage P1 Doc polypeptide, as described by Jensen et al., 1995.

It will be understood that in this context, the term "functional equivalent" includes variants or derivatives of any of the above first kind of polypeptides the sequences of which have been modified by substitution, deletion or addition of one or more amino

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acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

In accordance with the invention, the *relE* family gene or any gene coding for a toxin
5 of a proteic killer system is provided in the microbial cells at a location where it can be expressed effectively. Thus, in useful embodiments the gene is present on the chromosome of the cells whereas in other embodiments it is preferably located on an extrachromosomal element such as a plasmid or a cosmid. In a specific embodiment, the microbial cells according to the invention do not contain a gene coding for a second
10 type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.

However, in other useful embodiments, the microbial cells comprise a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the
15 functional equivalent hereof, the binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted. Such a counteracting second kind of polypeptide is, as it is mentioned above, also referred to herein as an antitoxin or an antidote for the cytotoxic polypeptide.

20 Although, in certain uses of the present method, it is preferred that the genes coding for both the toxic polypeptide and the antitoxin herefor is under the control of the same regulatory sequences, it may, in other uses, be advantageous that the gene coding for the second kind of polypeptide is operably linked to a different regulatable regulatory DNA sequence as defined above, permitting that the gene coding for the
25 second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

It will be appreciated that the genes coding for the toxin polypeptide and the antitoxin polypeptide, respectively can be present on the same replicon such as a plasmid or on
30 the chromosome, or they can be present on different replicons in the microbial cells.

A useful second kind of polypeptide is the *RelB* polypeptide derived from *E. coli* K-12 which i.a. binds effectively to the *E. coli*-derived RelE polypeptide. However, the regulation of the toxic effect of the first kind of polypeptide can also be based on pro-

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- viding in the cells a gene coding for a second kind of antitoxically active polypeptide that is functionally equivalent to the *E. coli* RelB polypeptide. Such a gene can be derived from the chromosome or another replicon of a Gram-negative bacterium including *Enterobacteriaceae* spp. such as *E. coli*, *Hemophilus* spp. such as *H. influenzae*,
- 5 *Vibrionaceae* spp. such as *V. cholerae*, *Pseudomonadaceae* spp., *Helicobacter* spp. such as *H. pylori*, and *Synechosystis* spp. belonging to the group of cyanobacteria. Additionally, genes coding for structural and functional equivalents of the *E. coli* RelB polypeptide can be isolated from Gram-positive bacteria including lactic acid bacterial species such as *Streptococcus* spp., *Bacillaceae* spp. such as *B. thuringiensis*, and
- 10 *Mycobacterium* spp. and from species belonging to *Arhae* such as *M. jannaschii* and *A. fulgidus*. Sequences for the *E. coli* RelB polypeptide and for equivalents isolated from the above organisms are listed in Table 1.6.

- Genes coding for functional equivalents of the *E. coli* K-12 RelB polypeptide which in
- 15 accordance with the invention can be used for containing microbial cells and replicons include the genes coding for the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide and the prophage P1 Phd polypeptide.

- It will be understood that in this context the term "functional equivalent" includes
- 20 variants or derivatives of any of the above second kind of polypeptides, the sequences of which have been modified by substitution, deletion or addition of one or more amino acids and the gene product of which has retained at least part of the function of the gene product of the non-modified sequence.

- 25 It is a significant objective of the present invention to provide the means of conditionally controlling the survivability of microbial cells that expresses one or more genes coding for a gene product of interest. In accordance with the invention such an objective is pursued for any type of gene products including enzymes such as proteases, enzymes which are effective in degrading carbohydrates such as starch degrading en-
- 30 zymes, lipid degrading enzymes and nucleases.

However, it is of particular interest to provide containment of microbial cells wherein the gene product of interest is selected from an immunologically active gene product,

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a gene product that is effective in degradation of an environmental pollutant and a pesticidally active product.

Accordingly, in such specific embodiments the microbial cells are cells which further
5 comprise a DNA sequence that is selected from a sequence coding for an immunologically active gene product, a sequence coding for a pesticidally active gene product and a sequence coding for a pollutant degrading gene product.

In the present context, the term "immunologically active gene product" is used to describe an epitope (antigenic determinant) from a pathogenic organism which, when it is administered to the body of a human or an animal, is capable of stimulating the formation of antibodies therein. A microbial cell as defined herein which contains one or more genes encoding such a gene product can be utilized in the preparation of live vaccines. In the immunization against several pathogens it is considered advantageous
10 to administer live vaccines as compared to killed organisms or antigenic fragments of the pathogen, since the level of immunity conferred by a live vaccine is frequently higher than that conferred by vaccines comprising killed pathogenic organisms or fragments thereof. Most currently used vaccines comprising viable epitope-containing organisms are either based on recombinant non-pathogenic organisms encoding the
15 epitope or they are based on attenuated pathogenic organisms. The cell advantageously contains a multiplicity of genes each of which codes for a specific immunologically active gene product.

However, up till now the use of live vaccines has been limited since it is difficult to
25 obtain the right combination of attenuation, viability and adequate immune response. Furthermore, the deliberate release of genetically engineered microorganisms to the body and to the external environment which is a result of the use of viable recombinant organisms as vaccines, is currently not allowed in any country for reasons of public concern as to the possible long-term environmental impact, in particular the risk
30 of permanent establishment of the GEMs in the environment.

The present invention provides advantageous means of circumventing these problems associated with the use of known GEM-based live vaccines by introducing into a viable epitope-containing cell the regulatably expressible gene coding for a cell toxic

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polypeptide as defined above. In particularly interesting embodiments, the invention provides, as a useful basis for a viable vaccine, the microbial cells as defined above whose expression is stochastically induced.

- 5 In useful embodiments of the invention, the cell which contains the DNA sequence coding for an immunologically active gene product further comprises means for transporting the epitope, when expressed, to the outer surface of the cell, i.e. translocating it across the cell membrane. Preferably such a translocation is obtained by inserting the gene coding for the epitope into a nucleotide sequence coding for an outer cell
- 10 surface polypeptide structure such as fimbriae which contains the fimbrillin protein, pili, flagellae or certain other surface proteins including as an example the OM protein found in *Streptococcus* species. By providing the cell with such a hybrid nucleotide sequence being expressible in the cell, the gene product hereof will be a fusion or hybrid protein comprising the epitope and the relevant cell surface structure.

- 15 A cell in which a fusion protein is expressed which comprises the epitope fused to a surface structure protein by which the cell can adhere to the mucosal cells of a body to which the cell is administered is considered to be particularly useful in that the epitope will become in close contact with the mucosa and thereby effectively stimulate a
- 20 protective immune response in the form of the excretion of secretory antibodies of the IgA and IgG classes.

- Furthermore, the adhesion of the epitope-carrying cell will ensure that the cell is retained in the human or animal body for a period of time which is sufficient to obtain
- 25 the desired immune response. It is considered that a satisfactory immunization typically may be obtained if the cell is present in sufficient numbers in a particular body environment such as the intestinal tract for a period in the range of 15-30 days, depending on the nature and the activity of the epitope expressed from the cell.

- 30 As it will be understood from the above description of the gene coding for the cell function-limiting toxic polypeptide and the DNA sequence regulating its expression, the present invention may provide useful means of providing live vaccines based on recombinant organisms which are immunologically effective and which can be used

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without the risk of undesired spreading of recombinant genes to the microflora of humans and animals or to the outer environment.

- In accordance with the invention, a useful cell for the preparation of a live vaccine is
- 5 one selected from a bacterial species which inherently contains an outer surface structure as mentioned above. Such species include as examples species of *Enterobacteriaceae* such as *Salmonella* and *E. coli* species, *Vibrionaceae* and *Pseudomonadaceae*. It will be understood that strains of such species which are particularly useful in the present invention as the basis of a live vaccine as defined above, are non-pathogenic
 - 10 strains or strains having a low pathogenicity.

- The epitope expressed by a cell as defined above may be an epitope derived from any pathogenic organism or agent the obtainment of immunity against which is desirably. Such pathogens include viruses, bacteria and eukaryotic organisms such as fungi,
- 15 yeast or protozoa.

- In commercially important embodiments, the microbial cell comprising the gene coding for a cytotoxic polypeptide contains a nucleotide sequence coding for a pesticidally active gene product. In this context, the term "pesticidally active gene product" is
- 20 used to denote a product which, when expressed in a cell being released to an environment where there is a need to reduce or eliminate the presence of pests that feed on plants, including insect pests, nematodes and vermins such as rodents or birds, is effective in respect of such pest control.

- 25 Such pests are currently controlled by the administration of toxic chemical pesticides to the infested environment, but recently various naturally occurring pesticidally active organisms including viruses, bacteria and fungi have been used as biological pest control products.

- 30 Prominent examples of such pesticidally active organisms include biotypes or strains of the species *Bacillus thuringiensis* that produce crystalline proteins being toxic to insects, in particular to caterpillars, and several viruses being pathogenic for insects in the larval stage or in the adult stage. However, the pesticidal effect of such organisms is frequently less satisfactory and there is a strong need in farming, forestry and horti-

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culture to provide improved pesticidally active organisms. One approach to solving this problem is to construct genetically engineered organisms having an increased toxic effect or a better survival rate in the environment. In addition to pesticidally active compounds from *B. thuringiensis*, such compounds are produced by other microbial organisms including *Bacillus sphaericus*, fungal species, algal species and plants. In accordance with the invention, genes coding for such biopesticides can be inserted and expressed in the biologically contained cells of the invention.

To the extent such improved organisms are developed, their use in the environment will, as a consequence of current public concern of the potential risks involved in deliberate release of such toxic or pathogenic GEMs, only be approved by official environmental agencies if it can be demonstrated that the release does not lead to an undesired propagation or to an extended survival of such organisms in the environment to which they are applied.

The present invention clearly provides the means of limiting the survival in the environment of genetically engineered pesticidally active organisms. As it has been explained above, the rate of expression of the cytotoxic polypeptide can be regulated stochastically and thus the survival rate of pesticidally active cells may conveniently be adapted to any specific need. Also, the cell function-limiting effect of the toxic polypeptide may, in accordance with the present invention, be adjusted by selecting a first kind of polypeptide that has an appropriate cell function-limiting effect.

In another useful embodiment, the invention provides a cell in which the gene coding for a desired gene product is a sequence coding for a pollutant-degrading gene product. It is known that several xenobiotic compounds polluting the outer environment including soil and water can be degraded by microorganisms having an inherent capability of degrading these compounds. Obviously, the technology of genetic engineering provides means of providing improved organisms having an increased pollutant-degrading capacity or having the capacity to degrade a broad range of compounds, in particular hydrocarbons.

However, the public concern as mentioned above are also relevant in this context and accordingly, the present invention provides useful means of providing improved pollu-

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tant-degrading microbial cells, the survival of which can be controlled by regulating the expression of the first kind of polypeptide as it is defined above. In particularly preferred embodiments, the cell contains a gene coding for a pollutant-degrading gene product, the expression of which is induced by the presence of a pollutant degradable
5 by the cell.

In addition to the above desired gene products, the microbial cells according to the invention can express any desired gene product including pharmaceutically active products such as e.g. hormones, interleukines and antibiotically active peptides.

10

As mentioned above, the invention provides in a further aspect a method of confining an extrachromosomal replicon to a microbial cell population. Basically, the method comprises the steps of isolating or constructing a microbial cell containing a gene belonging to the *relE* gene family expressing a first kind of polypeptide that is toxic for
15 the cell and introducing into the cell the extrachromosomal replicon to be confined, which replicon contains a gene coding for a second kind of polypeptide acting as an antitoxin for said first kind of polypeptide, and cultivating the cells under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromo-
20 somal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

In preferred embodiments of such a method the cell population consists of cells that comprise a gene coding for a gene product of interest as defined above.

25

The above method of confining an extrachromosomal replicon is particularly useful when the replicon is a plasmid that naturally occurs in a host cell in a low copy number. Accordingly, the method is useful for confining a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10
30 such as the range of 1-5.

Microbial cells to which a replicon can be confined in accordance with the invention include Gram-negative bacterial species such as species belonging to *Enterobacteriaceae*, *Hemophilus*, *Vibrionaceae* and *Pseudomonadaceae* and Gram-positive bacterial

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species, fungal cells including yeast cells, animal cells including human cells and insect cells, and plant cells.

- In a still further aspect, the invention provides a method of post-segregationally stabilizing a plasmid in a microbial host cell population as described above. As it is mentioned above, the method comprises the steps of (i) inserting into the plasmid a gene coding for a first kind of polypeptide as defined herein and a gene coding for a second kind of polypeptide as also defined herein that is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded, (ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed, whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.
- 15 The invention also provides a recombinant microbial cell as defined above, comprising a gene coding for a first kind of polypeptide. Such a cell can be a bacterium of a Gram-negative bacterial species including *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp or it can be of a Gram-positive bacterial species such as a *Bacillus* species or lactic acid bacterial species, a fungal cell including a yeast cell, an animal cell including a human cell and an insect cell, and a plant cell.

- As also mentioned above, the invention pertains in another aspect to a method of limiting the survival of a cell population in a first or a second environment, which method comprises as the first step that the cells are transformed with a gene coding for a cytotoxic polypeptide, which gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides.

In a specific embodiment of such a method, the survival of the cell population is limited in a first environment in which the gene is expressed whereby the cell population

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is contained in said first environment. In another embodiment, the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

- 10 In a still further embodiment of the above method, the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a DNA sequence encoding an antitoxin repressor substance which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby as a result of said decay, the function of the cells of the population will be gradually limited.

- In yet another aspect of the invention, there is provided a method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises as the first step providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides.

- In one specific embodiment of such a method the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

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The invention also provides a method as defined above of stochastically limiting in an environment the survival of a cell population. Such a method is particularly useful in the containment of recombinant cells which are to be released to the outer environment or the animal or human body.

5

The invention will now be described in further details in the following examples and the drawings wherein

Fig. 1 illustrates *relB_{K-12}::lacZ* and *relE_{K-12}::lacZ* translational fusions. Shown are relevant parts of the *lacZ* reporter plasmids pKG4001 (carrying a *relB_{K-12}::lacZ* fusion) and pKG4002 (carrying a *relE_{K-12}::lacZ* fusion). Numbers to the right in the Figure indicate *lacZ* expression levels in Miller units. The low expression level of *relE::lacZ* in pKG4002 is, in part, due to the presence of an intact *relB* gene located on the plasmid. The *relB* gene product represses the *relBE* promoter c. 130-fold;

15

Fig. 2 illustrates in vitro translation of *relBE_{P307}*-carrying plasmids. Lane 1: pBR322; lane 2: pHA402 (pBR322-*relB*⁺); lane 3: pHA403 (pBR322-*relBE*⁺); lane 4: pBR322; lane 5: pHA100 (pBR322-E11 contains the P307 *relBE* genes in their natural context); lane 6: pKG325; lane 7: pHA110 (pBR325-*relB*⁺);

20

Fig. 3 shows the structure of expression plasmid pNDM220. The plasmid is a mini-R1 vector whose copy number is amplifiable at 42°C due to the insertion of the temperature inducible λ P_n promoter upstream of the replication control region. The plasmid also carries the *cl₈₅₇* temperature-sensitive allele of the *cl* repressor. Genes shown are *copB* (copy number control), *repA* (initiation of replication), *parM* and *parR* (plasmid stability loci), *bla* (β -lactamase) and *lacP*. The plasmid contains the *LacI* regulated pA1/O4/O3 promoter upstream of a multiple cloning site that contains unique *BamHI* and *EcoRI* restriction sites. Thus genes inserted downstream of the promoter are inducible with IPTG;

30

Fig. 4 illustrates cell killing by *relE_{K-12}* and anti-killing by *relB_{K-12}*. Shown are optical density at 450 nm and viable counts as function of time for strains MC1000/pMG223 (*relE*⁺) (A, B), MC1000/pMG223/pMG2201 (*relB*⁻ control plasmid) (C, D) and MC1000/pMG223/pMG2202 (*relB*⁺ plasmid) (E, F). At time zero, transcription of *relE*

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on plasmid pMG223 was induced by the addition of IPTG (1 mM). Filled symbols indicate that IPTG was added. As seen from (E) and (F), the presence of *relB* on a second plasmid counteracted *relE* mediated cell killing;

- 5 Fig. 5 shows the structure of expression plasmid pBAD33. The plasmid is a medium copy number pACYC-derived vector. The plasmid carries the arabinose inducible pBAD-promoter and the *araC* gene of *E. coli*. Thus upon addition of arabinose to pBAD33 containing cells, genes inserted downstream of pBAD are transcriptionally induced. Genes shown in the Figure are: pACYC-ori: origin of replication; CM(R): gene
10 encoding chloramphenicol acetyl transferase; *bla'*: truncated (nonfunctional) gene encoding β -lactamase; mRNA1 encodes AraC activator protein; pBAD: arabinose-inducible promoter;

- Fig. 6 A/B illustrates cell killing by $RelE_{P307}$ and anti-killing by $RelB_{P307}$. Shown are optical density at 450 nm (A,C) and viable counts (B, D) as a function of time for strains
15 MC1000/pHA810/pBR322 (A, B) or MC1000/pHA810/pHA110 (carrying *relB*_{P307}). At time zero, transcription of *relE*_{P307} on plasmid pHA810 was induced by the addition of arabinose (0.02%). Filled symbols indicate that arabinose was added. As seen from (C) and (D), the presence of *relB*_{P307} on a second plasmid counteracted *relE*_{P307} mediated cell killing;
20

Fig.7 shows maps of pHA705 and pHA715;

- Fig. 8 illustrates OD₄₅₀ of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);
25

Fig. 9 shows viable counts of MC1000/pHA-Sp2, MC1000/pHA705 and MC1000/pHA715 (+/- IPTG);

- Fig. 10 is the DNA sequence of the *relBE*_{Sp2} locus of *S. pneumoniae*;
30

Fig. 11 is a map of pHA-Sp2;

Fig. 12 is a map of pHAG33;

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Fig. 13 is a map of pHAG33-2;

Fig. 14 is a map of pHAG33-3;

5 Fig. 15 is a map of pHAG33-4;

Fig. 16 illustrates OD₄₅₀ of KT2440/pHAG33-2, KT2440/pHAG33-3 and KT2440/pHAG33-4 (+/- IPTG);

10 Fig. 17 shows viable counts of KT2440/pHAG33-2, KT2440/pHAG33-3 and KT2440/pHAG33-4 (+/- IPTG);

Fig. 18 is a map of pHA810;

15 Fig. 19 illustrates Glucose run-out, OD₄₅₀ of MC1000/pHA810;

Fig. 20 illustrates Glucose run-out, viable counts of MC1000/pHA810; and

Fig. 21 illustrates that RelE_{K12}, RelE_{P307} and RelE_{M1} inhibit translation *in vitro*

20

EXAMPLES

Materials and methods

25

(i) Bacterial strains

The *E. coli* K-12 strain MC1000 (Casadaban and Cohen, 1980) which contains a chromosomal copy of the *relBE* genes was used as the standard cloning strain and
30 when a chromosomal copy of the *relB* operon was required. The *E. coli* K-12 strain JS115 (*leu*, *thy*, *thi*, *supE*, Δ *relB*), which contains a deletion covering the entire *relB* operon was provided by Olle Karlström. The latter strain was used for the regulatory studies of *relBE*.

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(ii) Plasmids used

Plasmid pOU253 is a mini-R1 based translational fusion vector carrying the *lacZ* gene of pNM482 (Minton, 1984). The fusion vector is segregationally stable due to the presence of the *parA* system of plasmid R1 (Dam and Gerdes, 1994).

Plasmid pNDM220 is a low copy-number mini-R1 expression vector carrying a multiple cloning site (mcs) placed between the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) and two transcriptional terminators.

10

pNDM220 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12157.

15 Plasmid pBD2430 (+388 - +1899) is a pUC18 derivative carrying the complete *relBE* operon and gene *IV* located downstream of *relF* (Olle Karlström, unpublished). The relevant *E. coli* DNA present in pBD2430 is shown in Table 1.1 below.

pBD2430 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12161.

20

(iii) Plasmids constructed

25 pKG4001: pBD2430 was digested with *EcoRI* and *XhoI* and the fragment carrying the *relB* promoter (Table 1.1) was inserted into pOU253 producing an in-frame translational fusion between *relB*_{K-12} and *lacZ*. Thus, pKG4001 carries a *relB*_{K-12}::*lacZ* translational fusion.

30 pOU253 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12158.

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pKG4002: pBD2430 was digested with *EcoRI* and *Bst1107I* and the resulting fragment was inserted into pOU253 producing an in frame translational fusion between *relE_{K-12}* and *lacZ*. Thus pKG4002 carries an intact *relB_{K-12}* gene and a *relE_{K-12}::lacZ* translational fusion.

5

pMG223: *relE_{K-12}* was amplified by PCR on pBD2430 with primers *relE1B* (5'-CCCCGGATCCATAAGGAGTTTTATAAATGGCGTATTTTCTGGATTTTGACG, SEQ ID NO:1) containing the *parA* Shine & Dalgarno (Gerdes and Molin, 1986) and *relE2* (5'-CCCCCCTCGAGGTCGACTCAGAGAATGCGTTTGACCGC-3', SEQ ID NO:2). The resulting *relE_{K-12}* carrying fragment was inserted into pNDM220 using the *BamHI* and *SalI* restriction sites. Plasmid pMG223 expresses *RelE_{K-12}* upon addition of IPTG.

10

pMG2201: this plasmid contains the *EcoRI-Eco47III* fragment from pBD2430 inserted between the *EcoRI* and *SalI* sites of pBR322. Plasmid pMG2201 carries the *relB_{K-12}* promoter and the 5' part of the *relB_{K-12}* gene.

15

pMG2202: pBD2430 was digested with *EcoRI* and *Bst1107I* and the *relB_{K-12}*-carrying fragment was inserted into pBR322 *EcoRI-SalI*. The resulting plasmid carries the *relB* promoter and *relB_{K-12}*.

20

PHA100: Plasmid pNZ945 is a pBS(+) derivative that carries a 4.3 kb *EcoRI* fragment from plasmid P307. This fragment encodes the RepFIB replicon and the *relBE* genes of P307 (Saul et al., 1989). The 4.3 kb *EcoRI* fragment (designated E11) of pNZ945 was purified and restricted with *PstI*. The resulting 2.2 kb *EcoRI-PstI* fragment was inserted into pBR322 restricted also with *EcoRI* and *PstI*. The pBR322-derived plasmid carrying the 2.2 kb *EcoRI-PstI* fragment was designated pHA100. Plasmid pHA100 codes for the entire *relBE* system from P307.

25

pNZ945 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession No. DSM 12160.

30

pHA110: The 2.2 kb *EcoRI-PstI* fragment of pHA100 was purified and digested with *ApoI* (*EcoRI* isoschizomer). The resulting *EcoRI-ApoI* DNA fragment (+1 to +1122)

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was inserted into the *EcoRI* site of pKG325 which was constructed as follows: Plasmid pBR325 was restricted with *PstI*, which has a unique recognition site in the plasmid. The resulting vector DNA fragment was made blunt ended with T4 DNA polymerase according to the manufacturer's instructions, and religated. Transformants

5 that were resistant to chloramphenicol and tetracycline, but sensitive to ampicillin were selected. Thus, pKG325 is a TcR, CmIR and ApS derivative of pBR325.

pKG325 was deposited on 30 April 1998 under the Budapest Treaty with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the accession

10 No. DSM 12159.

Plasmid pHA110 contains the *relB* promoter (*prelB*_{P307}) and gene *relB*_{P307}.

pHA205: Plasmid pHA205 is a derivative of the low copy-number mini-R1 expression

15 vector pNDM220 that contains the *relB* gene from P307. The PCR fragment generated from pNZ945 using primers RelB-P307/1: 5'-

CCCCCGGATCCCAGTCTTGAAAGGTGGC-3' (SEQ ID NO: 3) and RelB-P307/2: 5'-CCCCGAATTCTCATAGGTATTTATCCAG-3' (SEQ ID NO:4) was restricted with *Bam*HI and *Eco*RI and inserted downstream of the pA1/04/03 promoter of pNDM220.

20 pHA210: Gene *relE*_{P307} was PCR-amplified from pNZ945 with the primers: *relE*-p307/3 (5'-CCCCGGATCCAGATCTGGATAAATACC, SEQ ID NO:5) and *relE*-P307/2 (5'-CCCCGAATTCGTAACCTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA210 (+1089 to +1417) is thus a mini-R1

25 derivative carrying a pA1/04/03::*relE*_{P307} gene fusion which renders *relE*_{P307} inducible with IPTG.

pHA215: Genes *relBE*_{P307} were PCR-amplified from pNZ945 with the primers RelB-P307/1 (5'-CCCCCGGATCCAGTCTTGAAAGGTGGC, SEQ ID NO:3) and *relE*-P307/2

30 (5'-CCCCGAATTCGTAACCTTTCTGTGTTTATTGC, SEQ ID NO:6). The resulting PCR-generated DNA fragment was restricted with *Bam*HI and *Eco*RI and inserted into pNDM220 also restricted with *Bam*HI and *Eco*RI. Plasmid pHA215 (+840 to +1417) is thus a mini-R1 derivative carrying a pA1/04/03::*relBE*_{P307} gene fusion rendering the *relBE*_{P307} genes inducible with IPTG.

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pHA402: A *Pst*I-*Aat*II fragment from plasmid pHA205, which carries *lac*^R and the pA1/O4/O3::*relB*_{P307} gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA402 contains a *relB*_{P307} gene which is inducible with IPTG.

5

pHA403: A *Pst*I-*Aat*II fragment from plasmid pHA215, which carries *lac*^R and the pA1/O4/O3::*relBE*_{P307} gene fusion was inserted into pBR322 also restricted with *Pst*I and *Aat*II. Thus, the high copy-number plasmid pHA403 contains the *relBE*_{P307} genes which can be conditionally induced by the addition of IPTG.

10

pHA810: A DNA fragment encoding *relE*_{P307} was generated by PCR using primers *relE*-P307/4 (5'-CCCCCGAGCTCAGATCTGGATAAATACC, SEQ ID NO:7) and *relE*-P307/5 (5'-CCCCCGCATGCGTAACTTTCTGTGTTTATTGC, SEQ ID NO:8). The fragment was digested with *Sac*I + *Sph*I and inserted into the expression plasmid pBAD33 also digested with *Sac*I + *Sph*I. The resulting plasmid, pHA810 (+1089 - +1417), contains the pBAD::*relE*_{P307} gene fusion that renders *relE*_{P307} inducible with arabinose.

15

An overview of the bacterial strains and plasmids used herein is shown in Table 0.1 below.

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Table 0.1. Bacterial strains and plasmids

Strains	genotypes	Reference/Source		
5 MC1000	<i>Δlac leu ara</i>	Casadaban & Cohen, 1980		
JS115	<i>ΔrelB leu thy thi supE</i>	J. P. Bouche, unpublished		
Plasmids	Replicon	Resistance ^{a)}	<i>relBE</i> co-ordinates ^{b)}	Reference/Source
10 pOU253	mini-R1	Ap ^R	none	lab. collection
pBAD33	pACYC	Cml ^R	none	Guzman et al., 1995
pNDM220	mini-R1	Ap ^R	none	Gotfredsen & Gerdes, 1998
pBR322	ColE1	Ap ^R , Tc ^R	none	Bolivar et al., 1978
pKG325	pBR325	Tc ^R	none	lab. collection
15 pBD2430	pUC	Ap ^R	+388 - +1899	Olle Karlström collection
pNZ945	pUC	Ap ^R	+1 - +4298	Saul et al., 1989
pKG4001	mini-R1	Ap ^R	+388 - +596	Gotfredsen & Gerdes, 1998
KG4002	mini-R1	Ap ^R	+388 - +921	Gotfredsen & Gerdes, 1998
pHA100	pBR322	Tc ^R	+1 - +2198	Grønlund & Gerdes, 1998
20 pHA110	pBR325	Tc ^R	+1 - +1122	Grønlund & Gerdes, 1998
pHA205	mini-R1	Ap ^R	+840 - +1111	Grønlund & Gerdes, 1998
pHA210	mini-R1	Ap ^R	+1089 - +1417	Grønlund & Gerdes, 1998
pHA215	mini-R1	Ap ^R	+840 - +1417	Grønlund & Gerdes, 1998
pHA402	pBR322	Tc ^R	+840 - +1111	Grønlund & Gerdes, 1998
25 pHA403	pBR322	Tc ^R	+840 - +1417	Grønlund & Gerdes, 1998
pHA810	pACYC	Cml ^R	+1089 - +1417	Grønlund & Gerdes, 1998
pMG223	mini-R1	Ap ^R	+733 - +1020	Gotfredsen & Gerdes, 1998
pMG2201	pBR322	Tc ^R	+388 - +597	Gotfredsen & Gerdes, 1998
30 pMG2202	pBR322	Tc ^R	+388 - +921	Gotfredsen & Gerdes, 1998

a) Tc^R, tetracycline resistance; Ap^R, ampicillin resistance; Cml^R, chloramphenicol resistance.b) Co-ordinates refer to Table 1.1 (*relBE*_{K-12}, pMG-plasmids) or Table 1.2 (*relBE*_{P307}, pHA-plasmids)

(iv) Growth media and antibiotics

35

The growth medium was LB medium (Bertani, 1951) or A+B minimal medium (Clark and Maaløe, 1967) supplemented with 0.2% glucose and 1% casamino acids. For growth on solid media, LA-plates were used. LA is LB containing 15 g agar per litre. All media were supplemented with 50 µg/ml thymine for growth of the strain

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JS11507-05-99 Δ relBEF_{K-12}. Antibiotics were added at the following concentrations: ampicillin, 30 μ g/ml, and tetracycline, 10 μ g/ml. When indicator plates were used X-gal (5-Bromo-4-chloro-3-indolyl- β -D-galactoside) was added to a final concentration of 40 μ g/ml.

5

(v) Conditions of cell growth.

Cells were diluted in LB + antibiotics from an overnight culture to an OD₄₅₀ of 0.005.

The cultures were then grown at 37°C until an OD₄₅₀ of 0.4 and then diluted to an

10 OD₄₅₀ of 0.01 in 37°C LB containing 1 mM IPTG and antibiotics. Samples for OD₄₅₀ measurements and viable counts were taken at the time points indicated. Viable counts were made by plating dilutions of the cultures onto LA plates containing the proper antibiotics.

15 (vi) Coupled *in vitro* transcription and translation.

The reactions were performed using the *E. coli* S30 Extract System For Circular DNA as described by the supplier (Promega Corp.). 4 μ g of DNA was used in all reactions.

The reactions were run on a 16% Tricine-SDS-PAGE gel essentially as described by

20 Schägger and von Jagow (1987).

(vii) β -galactosidase assays.

β -galactosidase assays were performed essentially as described by Miller (1972).

25

(viii) Homology search.

BLAST searches were performed at the GENESTREAM BLAST network server CRBM Montpellier, France. Standard conditions were used except that the blosum 80 matrix

30 was used.

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EXAMPLE 1

The occurrence of *relBE* operons in bacteria and *Archae*5 1.1. Nucleotide sequence of the *relBE* operon of *E. coli* K-12

The DNA sequence of the *relBE* operon from *E. coli* K-12 is shown in Table 1.1. In this Table the transcriptional start site of the *relBE* mRNA is indicated with two asterisks (heterogeneity). IR indicates inverted repeats in the promoter and terminator regions.

10 Start codons and stop codons are shown in bold. The transcriptional termination point (*ttp*) of the *relBE* mRNA is also indicated with a vertical arrow. The DNA sequence is from Bech et al., 1985.

By visual inspection of the *relB*_{K-12} and *relE*_{K-12} genes there was found striking similarity
15 with the so-called "proteic plasmid stabilization systems" as described by Jensen and Gerdes (1995). First, *relE*_{K-12} codes for a very basic protein (RelE_{K-12}; pI = 9.7) of 95 amino acids (aa), and *relB*_{K-12} codes for a very acidic protein (RelB_{K-12}; pI = 4.8) of 79 aa.

20 The sequences of proteins RelB_{K-12} and RelE_{K-12} are shown in Tables 1.5 and 1.6, respectively. These Tables show multiple sequence alignments of the RelB and RelE gene families. Conserved amino acids at a given position are shown with shading as follows: two amino acids are considered conserved if they both belong to one of the following groups: group 1: D and N; group 2: E and Q; group 3: S and T; group 4: K
25 and R; group 5: F, Y and W; group 6: L, I, V and M. Light grey shading indicates 60-80% conservation, dark grey indicates 80-99% conservation and black indicates 100% conservation. Note in Table 1.6 the fully conserved glycine at position 69 (G in consensus line) and the fully conserved arginine at position 79 (R in consensus line). The entrez database accession numbers of the protein sequences are given in Tables
30 1.3 and 1.4.

The *relB*_{K-12} and *relE*_{K-12} genes are co-transcribed with a third gene, *relF* (also denoted *orf-3* or *hokC*), which is homologous to the *hok* gene from plasmid R1 (Gerdes et al., 1986). The start site (i.e. the 5'-end) of the *relBE* mRNA was determined to be 31 nu-

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cleotides upstream of the *relB*_{K-12} AUG start-codon (Bech et al., 1985) and was confirmed (M. Gotfredsen and K. Gerdes, 1998). Inverted arrows in the *relBE* promoter region (Table 1.1) indicate putative binding sites for regulators of transcription (i.e. the *RelB*_{K-12} and *RelE*_{K-12} proteins themselves).

5

The properties described above suggested that *RelE* could be a cytotoxin and that *RelB* could be an antitoxin which counteracts the toxicity elicited by *RelE*.

Table 1.1. DNA sequence of the *relBE* operon from *E. coli* K-12 (SEQ ID NO:9)

10

1 CTTAATTTCA GGCCCCATCG GATCACACAT GGAGAGTTTT TATGAATAAC
51 CCCGTCTGTC TTGATGACTG GTTGATTGGC TTTAAAAGCT TGTTGACAGG
101 GGTAACCGTT CGGCAATAAT TTTCTGCCGC ATGCGGGTGT TGCATAAAAC
151 GTGTTACGTT CCTTTATCGA CAGGTCAGGT CACCGCTCAC CCGCCGACGA
201 GAAAGCAACA CTGACATGCT AAAGCAAAA ATAGATGAAT AAGTTGAGTT

15

251 GTGCATATGT AGCCTGACCG TCACAAAGTA TATGGTGTCT GTACCAGTAA

20

301 GATGATGGCC GGACTCTTTA AAAACGAGCT GACCTGCACA ATACAGGATG

351 GACTTAGCAA TGGCTGCTCC TGGCACAAAG CGGACAGTGA TCACCGTTCT

401 TACGACTACT TTCTGACTTC CTTCGTGACT TGCCCTAAGC ATGTTGTAGT

25

451 GCGATACTTG TAATGACATT TGTAATTACA AGAGGTGTAA GACATGGGTA
 **→*relBEF* mRNA *relB* start
 ---->IR<-----

30

501 GCATTAACCT GCGTATTGAC GATGAACTTA AAGCGCGTTC TTACGCCGCG

551 CTTGAAAAAA TGGGTGTAAC TCCTTCTGAA GCGCTTCGTC TCATGCTCGA

601 GTATATCGCT GACAATGAAC GCTTGCCGTT CAAACAGACA CTCCTGAGTG

35

651 ATGAAGATGC TGAACCTGTG GAGATAGTGA AAGAACGGCT TCGTAATCCT

701 AAGCCAGTAC GTGTGACGCT GGATGAACTC TGATGGCGTA TTTTCTGGAT
 End *relB* Start *relE*

40

751 TTTGACGAGC GGGCACTAAA GGAATGGCGA AAGCTGGGCT CGACGGTACG

801 TGAACAGTTG AAAAGAAGC TGTTGAAGT ACTTGAGTCA CCCCAGATTG

45

851 AAGCAAACAA GCTCCGTGGT ATGCCTGATT GTTACAAGAT TAAGCTCCGG

901 TCTTCAGGCT ATCGCCTTGT ATACCAGGTT ATAGACGAGA AAGTTGTCGT

951 TTTCGTGATT TCTGTTGGGA AAAGAGAACG CTCGGAAGTA TATAGCGAGG

50

1001 CGGTCAAACG End *relE*
 CATTCTCTGA ACCAAAGCAT GACATCTCTG TTTTCGCACCG

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```
Start hokC (relF)
```

1051 AAGGTGACAC TTCTGCTTTG CGTTGACAGG AGAAGCAGGC **TATGAAGCAG**

1101 CAAAAGGCGA TGTTAATCGC CCTGATCGTC ATCTGTTTAA CCGTCATAGT

1151 GACGGCACTG GTAACGAGGA AAGACCTCTG CGAGGTACGA ATCCGAACCG

End · hokC

1201 ACCAGACGGA GGTCGCTGTC TTCACAGCTT ACGAACCTGA GGAGTAAGAG

1251 ACCCGGCGGG GGAGAAATCC CTCGCCACCT CTGATGTGGC AGGCATCCTC

1301 AACGCACCCG CACTTAACCC GCTTCGGCGG GTTTTGT TTATTTTCAA

--- IR --- - tít

1351 CGCGTTTGAA GTTCTGGACG GTGCCGGAAT AGAATCAAAA ATACTTAAGT
(data base accession number X02405)

[illegible]

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Table 1.3. *relE* homologues from Gram-positive and Gram-negative bacteria and Archaeae

5	Bacterial species	entrez accession	gene ^{a)}	Number of aa	MW (kD)	pI
Gram-negative bacteria:						
10	<i>E. coli</i> K-12	132284	<i>relE</i> _{K-12}	95	11.2	9.7
	<i>E. coli</i> K-12	984581	<i>relE</i> _{SOS} ^{b)}	92	10.8	9.5
	<i>E. coli</i> plasmid P307	516611	<i>relE</i> _{P307}	95	11.2	9.9
15	<i>H. influenzae</i>	1175293	<i>relE</i> _{Hi}	102	11.9	6.7
	<i>V. cholera</i>	396846	<i>relE</i> _{Vc}	96	11.2	9.9
20	<i>H. pylori</i>	2314031	<i>relE</i> _{Hp}	88	10.4	7.9
	<i>Synechosystis</i>	1653777	<i>relE</i> _{Sy}	120	13.7	7.9
Gram-positive bacteria:						
25	<i>B. thuringiensis</i>	520407	<i>relE</i> _{Bt}	74	8.6	9.7
	<i>M. tuberculosis</i> #1	2612811	<i>relE</i> _{Mt1}	87	10.2	11.0
	<i>M. tuberculosis</i> #2	2695832	<i>relE</i> _{Mt2}	97	11.1	9.5
30	Archae:					
	<i>M. jannaschii</i> #1	1498833	<i>relE</i> _{Mj1}	90	11.0	10.2
	<i>M. jannaschii</i> #2	1499953	<i>relE</i> _{Mj2} (*)	88	10.6	10.0
35	<i>M. jannaschii</i> #3	1591583	<i>relE</i> _{Mj3} (*)	91	11.1	10.1
	<i>A. fulgidus</i> #1	2648176	<i>relE</i> _{Af1}	87	10.6	10.3
40	<i>A. fulgidus</i> #2	2649499	<i>relE</i> _{Af2}	92	11.0	9.9
	<i>A. fulgidus</i> #3	2649496	<i>relE</i> _{Af3}	85	10.0	10.0
	<i>A. fulgidus</i> #4	2649514	<i>relE</i> _{Af4}	86	10.2	9.9

45 a) *relE* homologues marked with (*) are not located adjacent to a *relB* partnerb) The *relBE*_{SOS} system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994)

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Table 1.4. *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae*

5	Bacterial species	entrez accession	gene ^{a)}	Number of aa	MW (kD)	pI
Gram-negative bacteria:						
10	<i>E. coli</i> K-12	132283	<i>relB</i> _{K-12}	79	9.1	4.8
	<i>E. coli</i> K-12	984582	<i>relB</i> _{SOS} ^{b)}	86	9.4	5.2
	<i>E. coli</i> K-12	984588	<i>relB</i> _{K-12.2} (*)	97	11.2	5.5
15	<i>E. coli</i> plasmid P307	516610	<i>relB</i> _{P307}	83	9.2	4.4
	<i>S. typhimurium</i>	731639	<i>relB</i> _{St} (*)	68	7.6	5.3
20	<i>H. influenzae</i>	1573712	<i>relB</i> _{Hi}	98	11.0	4.7
	<i>V. cholera</i>	396847	<i>relB</i> _{Vc}	82	8.9	4.4
	<i>H. pylori</i>	2314037	<i>relB</i> _{Hp}	95	11.4	9.8
25	<i>Synechosystis</i>	1653776	<i>relB</i> _{Sy}	86	9.9	4.7
Gram-positive bacteria:						
30	<i>B. thuringiensis</i>	520406	<i>relB</i> _{Bt}	85	10.1	4.5
	<i>M. tuberculosis</i> #1	2612810	<i>relB</i> _{Mt1}	93	10.2	4.6
	<i>M. tuberculosis</i> #2	2695833	<i>relB</i> _{Mt2}	89	9.8	5.1
Archae:						
35	<i>M. jannaschii</i> #1	1498832	<i>relB</i> _{Mj1}	82	9.6	4.5
	<i>A. fulgidus</i> #1	2648190	<i>relB</i> _{Af1}	65	7.8	4.8
40	<i>A. fulgidus</i> #2	2649516	<i>relB</i> _{Af2}	62	7.4	4.3
	<i>A. fulgidus</i> #3	2649510	<i>relB</i> _{Af3}	72	8.5	4.5
45	<i>A. fulgidus</i> #4	269513	<i>relB</i> _{Af4}	57	6.7	4.1

a) *relB* homologues marked with (*) are not located adjacent to a *relE* partner.b) The *relBESOS* system of *E. coli* K-12 contains a LexA binding-site in the promoter region (Lewis et al., 1994).

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Table 1.5. Alignment of *relE* homologues from Gram-positive and Gram-negative bacteria and *Archae* (SEQ ID NOS:10-26)

[illegible]

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Table 1.6. Alignment of *relB* homologues from Gram-positive and Gram-negative bacteria and *Archae* (SEQ ID NOS:27-43)

	5	10	15	20	25	30	35	40	45	50	55													
relB-SOS	----	MAANAF	RRARR	DDDLK	QNAADV	LAGMGLT	ISD	VRIT	LT	KVAREK	ALPFDL	----	REP	NGLT	IOSIKN	----	SE	66						
relB-VC	----	MTRI	ADAA	ST	TPKAN	PKVATS	AFGAP	VAV	IN	----	RNEP	AFYCV	----	ASTY	IMDK	LED	----	LE	61					
relB	----	MPNI	ILSD	TSAS	----	SKIN	PMAT	VSAG	DGFF	VAI	IN	----	----	----	----	----	QE	61						
relB-Mt1	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Mt2	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-K12_2	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-St	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-coli	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-HI	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-MJ	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Af1	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Af3	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Af2	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Af4	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-Hp	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-synec	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-BT	----	MRIL	PIST	IKG	INEFT	DA	SSQD	QIT	TNG	GAPAA	VGAD	----	EWES	LOET	LYW	LAD	----	IA	72					
relB-SOS	----	AGID	VHK	AKD	ADD	L	FDK	LGI	----	----	----	----	----	----	----	----	----	----	86					
relB-VC	----	LLA	I	A	K	E	R	L	S	E	D	S	V	S	N	I	D	D	----	82				
relB	----	L	V	K	L	V	A	E	R	S	N	Q	P	L	H	D	V	D	K	Y	L	----	83	
relB-Mt1	----	S	G	R	T	Y	G	E	D	E	I	R	A	E	F	G	V	P	R	A	P	H	----	93
relB-Mt2	----	A	G	R	F	V	S	N	D	E	I	R	N	R	Y	T	A	R	----	----	----	----	89	
relB-K12_2	----	E	I	T	R	A	E	Q	V	L	N	D	T	D	D	F	N	D	F	K	E	----	97	
relB-St	----	E	F	A	I	M	A	V	H	G	N	E	----	----	----	----	----	----	----	----	----	68		
relB-coli	----	L	V	E	I	V	K	E	R	L	R	N	P	K	P	V	R	T	L	D	E	L	----	79
relB-HI	----	F	F	I	E	A	S	E	N	Y	S	A	E	F	T	R	I	L	N	G	Q	----	98	
relB-MJ	----	K	S	L	D	K	K	E	T	V	P	A	E	A	L	K	E	L	G	L	----	82		
relB-Af1	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	72		
relB-Af3	----	E	V	K	L	F	E	D	I	----	----	----	----	----	----	----	----	----	----	----	----	----	72	
relB-Af2	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	72	
relB-Af4	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	----	72	
relB-Hp	----	D	T	K	N	R	L	C	F	K	S	I	E	D	F	K	K	C	E	N	L	----	95	
relB-synec	----	E	E	Y	K	O	G	R	I	P	V	E	N	W	E	G	I	D	V	E	----	86		
relB-BT	----	E	G	V	S	G	E	D	A	K	R	E	F	G	L	I	D	L	P	----	----	----	85	

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1.2. Nucleotide sequence of the *relBE* operon of plasmid P307

5 genes described above.

The DNA sequence of the *relBE*_{P307} genes is shown in Table 1.2. The transcriptional start site of the *relBE* mRNA is indicated with an asterisk, and the -10 and -35 sequence elements of the *relBE* promoter are underlined. The Shine & Dalgarno sequence of the *relB* and *relE* genes are doubly underlined. The DNA sequence is from Saul et al., 1989.

Again, *relE*_{P307} codes for a very basic protein of 95 aa (pI = 9.9), and *relB*_{P307} codes for a very acidic protein of 83 aa (pI = 4.4), see Tables 1.3 and 1.4. The protein sequences of RelE_{P307} and RelB_{P307} are also shown in Tables 1.5 and 1.6, respectively. The start site (i.e. the 5'-end) of the *relBE*_{P307} mRNA was determined to be located 27 nucleotides upstream of the *relB*_{P307} AUG start codon. Inverted arrows in the *relBE*_{P307} promoter region (Table 1.2) indicate putative binding sites for regulators of transcription (i.e. the RelB_{P307} and RelE_{P307} proteins).

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Table 1.2. DNA sequence of the *relBE* operon from the *E. coli* plasmid P307 (SEQ ID NO:44)

5 301 GAGTATCATA TTAGGATACG GGTGGGTGAC GCCCACCTCT GGCATAGAAC
 351 GGACATTCAT TGATGCCATG CCAGAATGGA CGTTCAGGTT ATTCCGTCCA
 401 GTTCTGCTGG CAACGCGAGA TCTCCCCTGG TATAGTGATG CCACAGCAAA
 10 451 GCGCTCAAAC AGGGATAATA TGATGGAAAT CAAGGCTCAA CAGTTTTGTC
 501 ACATCAACGG GCGGCAAGT CCTTACTGAC AACGGACAAC AAGGTATGGG
 551 CGGCGTGGCG GGTATCGGTT CCACGACTGA AAAGCATCAG GGGCGCGTGG
 15 601 CGGAAGCGAT TTTTGCGAAC TGC GCGGAAC TGGATAACGA CCAGCTTAAC
 651 GAGATCATCG AGTGGGTTTCG GCTCTATCAG CGCTGAATGC CACTATCAGG
 20 701 CTGCGCAAGC GGCCTTTTTT ACGCCCCTTG TTTAATTCCC GCACTACCTG
 751 GACG TTCAGG TGATTCTGTC CATCTGTACA AAAACAATA AAAGACTTGT -35
 25 801 TAACAGGTCA TGTAAGGAGT ATCTTTGAGA CTGGTTAAAC AGTCTTGA
 SD start *relB* *→ *relBE*_{P307} mRNA
 851 GGTGGCCTAT GCCTAACATT ATTCTCAGTG ATACAAGCGC CAGTGTCAGC
 30 901 GAGCTGAAGA AAAACCCGAT GGCGACAGTC AGCGCCGGTG ATGGTTTCCC
 951 GGTGCTATC CTGAACCGTA ATCAGCCTGC TTTCTACTGT GTACCCGCGAG
 35 1001 AGCTGTACGA AAAGATGCTT GATGCCCTAG ACGATCAGGA GTTGGTTAAA
 SD
 1051 CTGGTAGCCG AACGCAGCAA CCAACCGCTG CATGATGTAG ATCTGGATAA
 40 end *relB*/start *relE*
 1101 ATACCTATGA GGTATCAGGT AAAATTCAGG GAAGATGCGC TGAAAGAGTG
 1151 GCAAAACTG GACAAGGCTA TTCAGCAACA GTTTGCGAAA AAGCTAAAAA
 45 1201 AGTGCTGTGA CAATCCGCAT ATTCCTTCCG CAAACTGCG TGGGATAAAG
 1251 GACTGCTACA AAATAAAATT ACGTGCCTCA GGTTCGCGC TGGTCTATCA
 1301 GGTGATTGAC GAACAATTAA TTATCGCTGT TGTAGCTGTG GGTAAACGTG
 50 end *relE*
 1351 AGCGCAGTGA CGTTTATAAT CTTGCCAGCG AAAGAATGAG ATAAAGCAA

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1401 TAAACACAGA AAGTTACTCT GCGTTATGG GGTAATGCAA AGTATGAGTC
1451 GTAGAGGGAA TTGCCTGGAT AATTCGCCGA TGGAAAGAGT CTTTCGCAGC
1501 CTTAAAAGTG AATGGCTTCC GAAAGGTGGT TATGGTGATT TTAGCCATGC
(database accession number M26308)

FOOTNOTES

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1.3. Nucleotide sequence and proteins of a *relBE* homologous operon from *Bacillus thuringiensis*

Using BLAST database searching (Altschul et al., 1990) it was found that transposon
5 Tn5401 from the Gram-positive organism *B. thuringiensis* contains, in one end or
asymmetrically located, a two-component system which exhibits both structural and
sequence similarity with the above described *relBE* systems from *E. coli*. This homol-
ogy is surprising given that it has not previously been described that *relBE*-like genes
are found in organisms other than *E. coli*.

10

The nucleotide sequence of the *relBE* operon from Tn5401 is shown in Table 1.7. In
this Table the transcriptional start-site of the *relBE* mRNA is indicated with an asterisk
(Baum, 1994). IR indicates inverted repeats in the *relBE_{Bt}* promoter region. Start co-
dons and stop codons are shown in bold. The Shine & Dalgarno sequence of the *relB_{Bt}*
15 gene is doubly underlined. The DNA sequence is from Baum et al., 1989.

The *relE_{Bt}* gene codes for a very basic protein of 74 aa (pI = 10.6) and the *relB_{Bt}* gene
codes for an acidic protein of 87 aa (pI = 4.4). The protein sequences of RelE_{Bt} and
RelB_{Bt} are aligned with the other RelE and RelB homologues in Tables 1.5 and 1.6, re-
20 spectively. The modular, structural and physico-chemical similarities between the *B.*
thuringiensis system and the *E. coli* systems suggested that the genes may exert simi-
lar functions in very different bacteria.

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Table 1.7. DNA sequence of the *relBE* operon from the Gram-positive organism *B. thuringiensis* (SEQ ID NO:45)

5 3701 CTCGTTTTTT CTGTTGGTAC AAACCTAATT GATTTTGAAT AATTTGTTTG
3751 TACCAGTCCT TTTTGCTTAG CCCAGTCAAA ATAACGTTTG ATTGAATTAA
3801 TGCGCCGGTT AATCGTAGAA GGTTTTAGTA ATCTTGTAAC TTGCATATGC
10 3851 CCTCGATATC GAGCAATAGT GCGAGCGGTA ACTTCTATTG GATGAAAAAG
3901 AGTATCCTCA GCATGTTTTT CCCACACATT TTCAAACCAA AATACAAAAT
3951 CTTTTAAATC ACTCGTATAT TCTTTTAGTG TTTTGTATG CAAATCTCCT
15 4001 TCTTGAGATA AGCTAGAAAT AAAATCGGAA ATCAAAGATG TTGCTTGAT
4051 AGAAATTGTT TTAGTGGAAT GCATAAATAC CTCCTCTTTT ATTGACTTAC -35
20 -10 *→ *relBE_{Bt}* mRNA
4101 ATTAGCGGAC ATGATATTTT AATCTTATCA ATTATGTTAG CGGACATCAA
25 4151 ACATTTATTT TCCCACACTT CATGTCCACT AATATTAATT AGTGGACATT
----- → IR ← -----
SD Start *relB*
4201 TAAAACTATC TCGAAAGTAG GTGTAACACA TGGCTATTTCG TAAAGATGAA
30 4251 TTGTATCGGT TAATTGATCA CCTGGATCAA CAAGATGAAA AAGCAGCATT
4301 TGACTTTTTA GAATTTCTTG TTCAACGGTC AAGAAGAAAA CCTAAAGAAT
35 4351 GGGAAAAAAT TGATATGGCA GATCCTGATC ATGAACCGCT GTCTACACAA
4401 GAGTTAGAAC AGTTAAACAG TGAAGAAGGA TATGTATCAG GGGAGGACGC
4451 AAAACGTGAA TTCGGACTAC AAATTGATTT ACCATAAGTC CGCGGTGAAA
45 End *relB* Start *relE*
4501 TTTATTGCAA AGCAAGAAAA AGGGATTCAA AAAAGAATTG CAGAAGGATT
4551 GAAGGGACTT CTTAAGATTC CTCCTGAAGG AGATATTAAA AGTATGAAAG
50 4601 GTTACACAGA ACTATATCGA TTACGGATTG GAACCTTTTCG AATTTTATTT

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4651 GAAATAAATC ATGATGAGAA AGTCATATAC ATACAAGCAA TTGGAAATCG

End relE

4701 TGGTGACATC TATAAATAAG GCAAACATGC ATTTTAAAA GAAAGGTCTT

4751 CTGAATCGAA GAACCTTCCT TTTTGTGTG CGAATAATGT CCGCTAATGC

10

4801 TTGTTGCGTG ATTCTGTTCC ATTGCTACAC ATACCCC

(database accession number U03554)

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1.4. The archaeon *Methanococcus jannaschii* encodes a *relBE* homologous system

Again using database searching it was found that the completely sequenced genome of the methanogenic archaeon *Methanococcus jannaschii* codes for three *relE* homologous genes, one of which are located just downstream of *relB* homologous genes. This finding was surprising since, in many respects, archaeal organisms are more similar to eukaryotes than to bacteria (e.g. in their macromolecular synthesis apparatuses).

- 10 The DNA sequence of the *relBE*_{Mj1} system is shown in Table 1.8. In this Table start codons and stop codons are shown in bold. The DNA sequence is from Bult et al., 1996.

- Gene *relE*_{Mj1} codes for a very basic polypeptide of 90 aa (pI = 11.0) and gene *relB*_{Mj1} codes for an acidic polypeptide of 82 aa (pI = 4.4). The aa sequences of the RelE_{Mj1} and RelB_{Mj1} proteins are aligned with the other RelBE homologues in Tables 1.5 and 1.6, respectively. Thus, these basic similarities suggested that the *relBE*_{Mj1} system may carry out similar or related functions in bacteria and archae. The properties of the second and third *relE* homologues of *M. jannaschii* are also given in Table 1.3. These comparisons show that *M. jannaschii* codes for one complete *relBE* homologous gene system and for two *relE* homologues without an adjacent *relB* partner.

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Table 1.8. DNA sequence of a *relBE* homologous gene system from the archaeon
Methanococcus jannaschii (SEQ ID NO:46)

5 751 CCGATACCGT TGCTGGAGAC ATAGCTGGAG CTTTGAAGGC GGAGAAGCTT.
801 ATTTTAATAA CAGATGTTGA TGAATAATG GATGATATAA ATAATCCAGA
851 GACGTTGCAT AGAAAATTAA CAGCTTCAGA ACTAAAAGAA ATGATAGAAG
10 901 ATGGAAGAAT AAAGGGAGGG ATGATTCCAA AGGCTGAAAG TGCCTTATAT
951 GCCTTAGAGC ATGGAGTTAA GAGCGTTCAT ATAATAAATG GAAAGATTCC
1001 TCATGCTTTG TTGTTGGAGA TATTACAGA GGAGGGTATT GGGACGATGA
15 1051 TAACAAGAGA TTAAAGTTTT TATATTATAA ACTACTTAAG AATTAAAATA
Start *relB_{Mj1}*:
1101 AGACAAATAA GGGGATAACT ATGCTCAATA TAAACAAAGA GATAGCACAA
20 1151 ATAGAACTG AATTGAATGA ATTGAAAAA TTGAGAGATG AAATCTCTGA
1201 AAGGATTGAA AAATTAGAAA TAAAGTTATT AAAATTGAAA GCATTAGCTA
25 1251 TTCCAGAGGA GGAATTTGAA GAGGATTATG AAGAAATTAT AGAAGATGTT
1301 AAAAAATCTC TGGATAAAAA AGAGACTGTG CCAGCAGAAG AGGCTTTGAA
End *relB_{Mj1}*/start *relE_{Mj1}*:
30 1351 AGAATTGGGA TTATTATGAA GTTAAACGTT GAGATACATA AAAGAGTCTT
1401 AAAAGATTTA AAGGATTTGC CTCCCTCAA CTTAAAGAAG TTAAAGAAC
1451 TAATAGAAAC ATTAAAAACC AATCCCATTC CAAAAGAAAA ATTTGATATT
35 1501 AAAAGATTAA AAGGCAGTGA TGAGGTTTAT AGAGTTAGAA TTGGAAAATT
1551 TAGAGTTCAA TATGTTGTTT TATGGGATGA TAGAATAATA ATAATTAGAA
40 End *relE_{Mj1}*:
1601 AGATAAGTAG AAGAGAAGGA GCTTATAAAA ATCCCTAAGC TATTAAAAAT
1651 TCTAATGGCT ACATTTTTAT ATCTCTTTTC TTAATTCAA TAGAAAAAAC
45 1701 AGATTCCGCT GATACCATGA TTATTCTTTT AGATTTAAAT GGAACAATAG
(database accession number U67464)

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1.5. *relBE* homologous genes are ubiquitous in prokaryotes

Further *relBE* homologous two-component systems were discovered. The corresponding RelB and RelE homologous proteins are aligned in Tables 1.5 and 1.6, respectively.

5 It appears that *relE* homologous genes are present in a wide variety of Gram-negative bacteria (*E. coli*, *H. influenzae*, *V. cholera*, *H. pylori* and *Synechosystis*), in Gram-positive bacteria (*B. thuringiensis* and *M. tuberculosis*) and in Archae (*M. jannaschii* and *A. fulgidus*). Most strikingly, the archaeon *A. fulgidus* contains four complete *relBE* homologous gene systems.

10

A number of features become evident from the alignments of the proteins (Tables 1.5 and 1.6) and from the properties listed in Tables 1.3 and 1.4. First, all RelE homologues are basic with pH's around 8-10 whereas the *RelB* homologues are acidic with pl's about 4-5. Secondly, the RelE proteins are in general slightly larger (90-120 aa) than the *RelB* homologues (70 - 80 aa). Thirdly, the start codons of the *relE* genes are juxtaposed or even overlap with the stop codons of the linked *relB* partner, thus indicating translational coupling of *relE* to *relB*. These properties suggest that the proteins could exert similar functions in very different organisms.

15

20 EXAMPLE 2

Demonstration of translation of the *relB_{K-12}* and *relE_{K-12}* genes

Using the low copy-number *lacZ* fusion vector pOU253 (Table 0.1) in frame gene fusions between *relB_{K-12}* and *relE_{K-12}* and the *lacZ* gene were constructed (see Materials and methods). Thus plasmid pKG4001 (+ 388 to + 596) carries a fusion between *relB_{K-12}* and *lacZ*, and pKG4002 (+ 388 to + 921) carries a fusion between *relE_{K-12}* and *lacZ*. The structure of the relevant parts of these reporter plasmids are shown in Fig. 1. When present in strain MC1000, both plasmids expressed significant amounts of galactosidase fusion proteins, indicating that genes *relB_{K-12}* and *relE_{K-12}* are translated (Fig. 1). The *relE_{K-12}-lacZ* fusion (pKG4002) expressed significantly lower amounts of β -galactosidase than the *relB_{K-12}-lacZ* fusion, mainly because pKG4002 encodes an intact *relB_{K-12}* gene which produces the RelB_{K-12} autorepressor which inhibits transcription from the *relB* promoter.

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EXAMPLE 3

Demonstration of translation of the *relB*_{P307} and *relE*_{P307} genes

- 5 To detect authentic RelB_{P307} and RelE_{P307} proteins, *in vitro* translation reactions were carried out using high copy number pUC-plasmids carrying genes *relE*_{P307} (pHA403), *relB*_{P307} (pHA402) or both genes (pHA100) (for construction of these plasmids, see Materials and Methods and Table 0.1). Proteins produced in the *in vitro* translation reactions were labelled with ³⁵S-Methionine and separated by SDS-page. Fig. 2 shows
- 10 the direct visualization of RelB_{P307} and RelE_{P307}, thus providing evidence that the corresponding genes are translated.

EXAMPLE 4

15 Demonstrating that *relE*_{K-12} is a cytotoxin

- The low copy-number cloning vector pNDM220 contains *lacIq* and the LacI regulated pA1/O4/O3 promoter (Lanzer and Bujard, 1988) upstream of a multiple cloning site (mcs). The genetic structure of pNDM220 is shown in Fig. 3. Without IPTG added to
- 20 the growth medium, the pA1/O4/O3 promoter is almost completely turned off. However, with IPTG, strong transcription is induced towards the cloning site. Therefore plasmid pNDM220 is suitable for the conditional expression of genes, in particular toxin-encoding genes.
- 25 The *relE*_{K-12} gene of *E. coli* K-12 (Bech et al., 1985) was PCR amplified and inserted into the mcs of pNDM220, resulting in pMG223 (for the construction of pMG223, see Materials and methods). Plasmid pMG223 (+ 733 to + 1020) was established in MC1000, which contains a chromosomal copy of the *relBE* operon. However, it was not possible to transform pMG223 into the JS115 strain, which carries a deletion of
- 30 the *relBE* operon ($\Delta relB$). Therefore, the induction experiments shown in Fig. 4 were accomplished using strain MC1000, which contains the chromosomal copy of *relBE*_{K-12}.

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Strain MC1000/pMG223 was grown in LB at 37°C. At time zero, IPTG was added to the growth-medium. After two hours of induction with IPTG, the viable counts decreased c. 600-fold (Fig. 4B). The decline started immediately and continued exponentially for about 2 hours. On plates containing IPTG, viable counts decreased even further (data not shown). The optical density (OD₄₅₀) increased during the first 20 minutes after addition of IPTG and then the culture became stationary (Fig. 4A). Addition of IPTG to growing cells containing the vector-plasmid had no effect (not shown). These results indicate that the *relE* gene encodes a cell toxin.

10 EXAMPLE 5

Demonstrating that RelB_{K-12} is an antitoxin

Plasmid pMG2202 (+388 to +921) is a pBR322 derivative that contains the *relB* gene expressed from its own promoter (see Table 1.1). Plasmid pMG2201 (+388 to +597) is a pBR322 derivative that contains the *relB* promoter and the first part of *relB*_{K-12}. Thus, pMG2201 does not contain an intact *relB* gene and was included in the analyses as a control plasmid. The strains MC1000/pMG223 (pA1/O4/O3::*relE*+) /pMG2202 (*relB*⁺) and MC1000/pMG223/pMG2201 (*relB*) were subjected to a physiological growth experiment similar to the one described in Example 4. As seen from Fig. 4E and 4F, the presence of the high copy-number *relB*-carrying plasmid suppressed *relE*-dependent cell killing. The antitoxin effect was dependent on an intact *relB* reading frame, since the control-plasmid (pMG2201) carrying the promoter region and the first part of the *relB* reading frame did not prevent the *relE* mediated cell killing (Fig. 4C, 4D).

EXAMPLE 6

Demonstrating that *relE*_{P307} encodes a very efficient cytotoxin

The medium copy number expression vector pBAD33 contains an arabinose inducible promoter (pBAD) with a multiple cloning site (mcs) and the *araC* gene (Guzman et al., 1995). The genetic structure of pBAD33 is shown in Fig. 5. Without arabinose added to the growth medium, the pBAD promoter is completely turned off. However, with

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arabinose, strong transcription is induced towards the cloning site. On top of this property, the pBAD promoter is repressible by the addition of glucose to the growth medium. Thus, by the addition of glucose, transcription from pBAD can be rapidly and efficiently turned off.

5

The glucose repression effect is epistatic to the inducer effect by arabinose. Hence, if cells with a pBAD-carrying plasmid are grown in a medium containing both arabinose and glucose then the promoter is not induced. However, if cell-growth depletes the medium for glucose, then the promoter will be induced. Therefore, plasmid pBAD33 is

10 suitable for the conditional turning on and off of the expression of genes, in particular toxin-encoding genes as described herein.

The *relE* gene of the *E. coli* plasmid P307 (Saul et al., 1989) was PCR amplified and inserted into the mcs of pBAD33, resulting in pHA810 (for the construction of plasmid

15 pHA810, see Materials and methods). Thus plasmid pHA810 contains the *relE*_{P307} gene inserted downstream of the pBAD promoter. Strain MC1000/pHA810 was grown in LB-medium without glucose at 37°C. At time zero, the culture was diluted into medium containing either 0 or 0.2% arabinose. In the arabinose-containing cul-

20 The decline continued exponentially throughout the experiment. After 240 min of induction with arabinose, viable counts had decreased more than five orders of magnitude. Without arabinose, cells containing pHA810 continued to grow exponentially (Fig. 6A and 6B, open symbols). On plates containing arabinose, none or very few viable cells were detected. These results show that *relE*_{P307} gene encodes an extremely

25 efficient cell toxin.

EXAMPLE 7

Demonstrating that RelB_{P307} is an antitoxin

30

Plasmid pHA110 (+1 to +1122) is a pBR322 derivative that contains the *relB*_{P307} gene expressed from its own promoter. The strain MC1000/pHA810/pHA110 (*relB*_{P307}) was subjected to a physiological growth experiment as described in Example 6. It appeared that the presence of the *relB*_{P307}-carrying plasmid pHA110 prevented

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*relE*_{P307} dependent inhibition of cell growth (Fig. 6C) and cell killing (Fig. 6D). This observation shows that *relB*_{P307} codes for an antitoxin that counteracts the cell killing caused by RelE_{P307}.

5 EXAMPLE 8

Determination of the frequency of spontaneous mutants that are resistant to the killing effect of RelE

- 10 Strain MC1000/pHA810 was grown exponentially to an OD of 0.5 and serial dilutions of the cell suspension were plated on LA plates containing chloramphenicol (selecting for plasmid pHA810) and with or without 0.02% arabinose (which induces expression of *relE* present in pAH810). On such plates without arabinose the plating efficiency of strain MC1000/pAH810 was normal, i.e. more than 99% of the viable cells produced
- 15 a colony. This indicated that the presence of pAH810 in itself had no effect on the viability of the cells. However, with arabinose the plating efficiency was reduced by about 10⁹ fold, thus indicating that expression of RelE is extremely toxic to the cells. The few surviving colonies that appeared eventually were retransformed with the RelE expression plasmid pHA210 which can co-exist with pAH810. However, none of the
- 20 surviving cells from the first round of selection (i.e. using pHA810) survived induction of RelE (by addition of IPTG) from the second plasmid pAH210.

These results show that resistance against RelE toxicity is a very rare event, as based on this experiment it is less than about 10⁻⁹.

25

EXAMPLE 9

Demonstrating that RelE of the Archeon *Methanoccus jannaschii* is toxic to *E. coli*.

- 30 The *relE* gene of *M. jannaschii* was amplified from genomic DNA using primers MJ-relE/2CWW (5'-CCCCGAATTCGCATGCGCCATTAGAAT, SEQ ID NO:47) and MJ-relE/1CW (5'-CCCCGGATCCGAGCTCGAGGCTTTGAAAGAATTGGG, SEQ ID NO:48). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into plasmid pNDM220 (Fig. 3) thus yielding pHA705 (Fig. 7). Similarly, *relB* and *relE*

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from *M. jannashii* were PCR amplified using primers relB-M.jannCW (5'-CCCCGGATC-CGTCGACGACAAATAAGGGGATAACTATG, SEQ ID NO:49) and MJ-relE/2CWW. The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220, thus yielding pHA715 (Fig. 7).

5

Plasmids pHA705 (carrying *relE*) and pHA715 (carrying *relBE*) were transformed into *E. coli* K-12 strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition of IPTG inhibited the growth of MC1000/pHA705 but not that of MC1000/pHA715, and Fig. 9 shows that viable

10 count was significantly reduced in the case of MC1000/pHA705 but not in that of MC1000/pHA715, thus demonstrating that RelE of *M. jannashii* is toxic to *E. coli*.

EXAMPLE 10

15 **Demonstrating that RelE of the Gram-positive bacterium *Streptococcus pneumoniae* is toxic to *E. coli*.**

Using BLAST database searching, we identified two homologues of the *relBE* genes of *S. pneumoniae*. The DNA sequence of the homologue designated *relE_{Sp2}* is shown in

20 Fig. 10. Gene *relE_{Sp2}* was PCR amplified from genomic DNA of *S. pneumoniae* strain RP46 using primers relE-Sp2/cw (5'-CCCCGGATCCGATGCATGATTAGGCTTGAAG, SEQ ID NO:50) and relE-Sp2/ccw (5'-CCCCGAATTCGAATGAAA-ATTACTTGAAAAAAG, SEQ ID NO:51). The resulting DNA fragment was cleaved with *Bam*HI and *Eco*RI and cloned into pNDM220 thus yielding plasmid pHA-Sp2 (Fig.

25 11).

Plasmid pHA-Sp2 (carrying *relE_{Sp2}*) was transformed into *E. coli* strain MC1000. Cells were grown exponentially and followed after the addition of IPTG. Fig. 8 shows that the addition of IPTG inhibited the growth of MC1000/pHA-Sp2, and Fig. 9 shows that

30 viable counts were dramatically reduced, thus demonstrating that expression of *relE_{Sp2}* is highly toxic to *E. coli*.

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EXAMPLE 11

Cloning of the *relE* genes of plasmid P307, *M. jannashii* and *E. coli* K-12 into the broad-host-range vector pHAG33.

5

The broad-host-range vector pVLT33 is an RSF1010 derivative that can be mobilized by an appropriate conjugation system (de Lorenzo, Eltis, L., Kessler, B. and Timmis, K.N. 1993. Analysis of *Pseudomonas* gene products using *lacI^P/Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123, 17-24). It also contains
10 the *tac*-promoter (*ptac*) and *lacI^P*. Since *ptac* is leaky and therefore unsuitable for the regulated expression of toxins, the promoter was replaced by the pA1/O4-O3 promoter of pNDM220. The resulting plasmid, pHAG33, is shown in Fig. 12. The *relE* genes of pHA210 (*relE_{P307}*), pHA705 (*relE_{Mj}*) and pMG223 (*relE_{K12}*) were cloned into pHAG33, resulting in plasmids pHA33-2 (Fig. 13), pHA33-3 (Fig. 14), and pHA33-4
15 (Fig. 15), respectively.

EXAMPLE 12

Demonstrating that RelEs of *E. coli* K-12, P307 and *M. jannashii* are toxic to *Pseudomonas putida*.

20

Plasmids pHA33-2 (*relE_{P307}*), pHA33-3 (*relE_{Mj}*) and pHA33-4 (*relE_{K12}*) were transformed into the *E. coli* K-12 strain S17-1. This strain contains the conjugation system of RP4 and is thus able to mobilize pHAG33-derived plasmids as described above (Simon et al.,
25 1986). After conjugation on solid medium to *P. putida* strain KT2440 according to standard procedure, strains KT2440/pHA33-2, KT2440/pHA33-3 and KT2440/pHA33-4 were established.

The strains were grown exponentially in LB containing 30 µg/ml ampicillin and 50
30 µg/ml kanamycin and followed after the addition of 2 mM IPTG. As seen from Fig. 16, the increment in cell-growth as measured by OD₄₅₀ was reduced by IPTG in all three cases. Furthermore, measurements of viable counts (Fig. 17) showed cell-killing in all three cases, most severe in the case of *relE_{K12}* (pHA33-4(+)) in Fig. 17). Thus, RelE

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proteins of P307 and *M. jannashii* are toxic to *P. putida* and RelE of *E. coli* K-12 is extremely toxic to *P. putida*.

EXAMPLE 13

5

Demonstrating biological containment by the depletion of a carbon source.

Plasmid pHA810 (Fig. 18) was constructed by inserting the *relE* gene of P307 into the expression vector pBAD33 (Fig. 5). The promoter (designated p_{BAD}) upstream of
10 *relE*_{P307} in pHA810 is repressed by glucose and induced by arabinose. The repression by glucose overrides induction by arabinose such that the simultaneous presence of glucose and arabinose in the growth medium results in repression of the promoter.

To simulate a realistic scenario in which the carbon source was depleted, we grew
15 MC1000/pHA810 in ABT minimal salts medium at 35°C in the presence of a limiting amount of glucose (0.025% w/v) (represses p_{BAD}) and varying the concentration of arabinose (induces p_{BAD}). Optical density (Fig. 19) and viable counts (Fig. 20) typical for such an experiment were obtained. As seen in Fig. 19, the rate of increase in OD₄₅₀ is severely reduced by the highest amounts of arabinose (0.050% and
20 0.075%). This was expected, since arabinose induces p_{BAD} and the limited amount of glucose (0.025%) cannot fully suppress p_{BAD} at high concentrations of arabinose. The glucose added (0.025%) was depleted by cell growth at an OD₄₅₀ = approx. 0.1. At this OD₄₅₀, a dramatic cell killing was seen in the case of 0.005%, 0.010%, and 0.025% of arabinose (Fig. 20). This result shows, that depletion of the carbon source
25 (glucose) leads to massive cell killing, and thus to biological containment of the plasmid that carries *relE*_{P307}.

EXAMPLE 14

30 Demonstrating that RelE of *E. coli* K-12 and *M. jannashii* are toxic to human cells.

The cell line 293 is a permanent line of primary human embryonal kidney cells transformed by human adenovirus type 5 (Ad 5) DNA (ATCC CRL-1573). The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and

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contain and express the transforming genes of AD 5 (Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59-74).

- 5 Plasmid pcDNA3.1(+) (Invitrogen) carries the constitutive promoter P_{CMV} from cytomegalovirus upstream of a multiple cloning site (mcs). Genes *relE* of *E. coli* K-12 and *M. jannashii* were inserted in the mcs, resulting in plasmids p5.4 and p5.3, respectively.
- 10 Plasmids pcDNA3.1(+)(control), p5.4 and p5.3 were transfected into cell line 293 by selection in medium containing G418 (geneticin), which selects for cells expressing the neomycin gene present on the plasmids. After 12 days, the cell density was measured by inspection. In the case of p5.4 (*relE*_{K-12}), between 0 and 5% of the cells had survived (as compared to the control). In the case of p5.3 (*relE*_{Mj}), between 5 and 15 10% of the cells had survived. These results indicate that the bacterial *RelE*_{K-12} and the archaeal *RelE*_{Mj} toxins both are lethal to human cells.

EXAMPLE 15

- 20 Demonstrating that *RelE*_{K-12}, *RelE*_{P307}, and *RelE*_{Mj} inhibit translation in vitro

DNA fragments comprising genes *relE*_{K-12}, *relE*_{P307} and *relE*_{Mj} were PCR amplified such that a T7 RNA polymerase promoter was placed upstream of the corresponding genes (according to Thisted et al., 1994).

25

The following primers were used: *relE*_{K-12} (P1: 5'-TGTAATACGACTCACTATAGATAAGGAGTTTTATAAATGGCGTATTTTCTGGATTTTG, SEQ ID NO:52) and P2 (CACCTTCGGTGCGAAACAG, SEQ ID NO:53); *relE*_{P307} (P3: 5'-TGTAATACGACTCATATAGATAAGGAGTTTTATAAATGAGGTATCAGGTAAATTCA (SEQ ID NO:54) and 30 P4: 5'-CTTTCCATCGGCGAATTATC, SEQ ID NO:55); *relE*_{Mj} (P5: 5'-TGTAATACGACTCACTATAGATAAGGAGTTTTATAAATGAAGTTTAACGTTGAGATAC SEQ ID NO:56) and P6: (5'-ATCATGGTATCAGCCGAATC, SEQ ID NO:57). T7 RNA polymerase sequences are underlined, and the strong Shine-Dalgarno (SD) sequence from the *parA* system of plasmid R1 is shown in italics.

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Using in vitro transcription with T7 RNA polymerase according to standard procedures, mRNAs encoding *relE*_{K-12}, *relE*_{P307}, and *relE*_M were produced and subsequently purified from a denaturing polyacrylamide gel. To facilitate the quantification of the mRNAs they were labelled with tritium (alfa-³H-CTP) during their synthesis. The *relE*-
5 encoding mRNAs (1.5 pmol) were used as templates in in vitro translation reactions employing an S30 extract (obtained from Promega) containing 150 µM of each amino acid except Methionine which was 1 µM.

Figure 21 shows SDS-PAGE (tricine-gel) analysis of such an experiment. The in vitro
10 translation reactions were initiated with unlabelled Methionine in order to produce RelE toxin in the reaction. Ten minutes after the addition of the unlabelled Methionine, radioactive 35-S-Methionine (5 pmol in a 15 µl volume) was added and the reaction continued for an additional 20 minutes. C in Fig. 21 denotes a control lane without exogenous mRNA added. The protein bands seen in this lane originate from translation
15 of mRNAs present in the S30 extract. In lane 1, the *in vitro* translation reaction contained an mRNA encoding the *relE* gene of *E. coli* K-12. As seen, the translation reaction was severely inhibited. In lane 2, a mRNA encoding a mutated *relE* (denoted *relEmE* and described in Gotfredsen et al, 1998) gene was added. As seen, the presence of this mRNA did not inhibit the reaction. This result shows that the RelE protein
20 produced during the initial incubation-period without 35-S-Met added inhibits the in vitro translation reaction (i.e. compare lanes 1 & 2). Furthermore, this lack of inhibition is correlated with loss of cell killing activity in vivo (since the mutated *relE* gene, *relEmE*, used in lane 2 is not toxic to *E. coli* cells), thus indicating that inhibition of translation is the actual cause of cell death in vivo. In lanes 3 and 4, mRNAs encoding
25 *relE* of plasmid P307 and the archaeon *M. jannashii* were added. As seen, the presence of these mRNAs inhibited the in vitro translation reactions as well. These results indicate that the RelE toxins from *E. coli* K-12, *M. jannashii* and plasmid P307 all act by inhibition of translation.

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EXAMPLE 16

Demonstrating that RelE_{K-12} is toxic to yeast cells.

5 1. Yeast strain

In these experiments the yeast strain *Saccharomyces cerevisiae* 281288DIV-36 (MATa his 4-5; LEU2 THR4 ura3-52 trp1 CYH2 KAR1) was used.

10 2. PCR amplification of RelE coding region

The RelE coding region was PCR amplified from the plasmid pMG223 using two oligonucleotide primers. The primer S-RelE was 24 nucleotides long (5'-TAGGTACCATGGCGTATTTTCTGG-3', SEQ ID NO:58). It contains *KpnI* and *NcoI* en-

15 donuclease restriction sites at the 5' end with an 8 nucleotide overhang. Primer AS-RelE was 23 nucleotides long (5'-GAGACCCCACACTACCATCGGCG-3', SEQ ID NO:59) and hybridises 400 nucleotides downstream the RelE termination codon and 392 nucleotides downstream the *EcoRI* site in plasmid vector pMG223. PCR amplifications were performed using Vent© Polymerase (New England Biolabs), 200
20 μ M of each dNTP. PCR reaction buffer (10 mM KCl; 10 mM (NH₄)₂SO₄; 20 mM Tris-HCl (pH 8.8); 2 mM MgSO₄; 0.1% Triton X-100) with 0.2 μ M of each of the primers. After 5 min denaturation (95°C) PCR was performed with 20 cycles, each cycle consisting of 1 min denaturation (92°C), 1 min primer annealing (50°C) and 1 min primer extension (72°C). Successful PCR products of 706 bp DNA fragments were
25 identified and purified from a 1% agarose gel after a run of 1 hour at 40 mA. The PCR product was digested with the two restriction enzymes *KpnI* and *EcoRI* and the fragment of 304 bp containing the RelE open reading frame was isolated after electrophoresis on a 1.2 % agarose gel, and purified using a gel extraction kit (Pharmacia).

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3. Cloning of the amplified *relE* gene

The isolated DNA fragment of the *relE* gene flanked with *KpnI* and *EcoRI* sites was ligated into the pYES2 expression vector (Invitrogen) previously digested with *KpnI* and *EcoRI* using standard procedures (Sambrook). After ligation, *E. coli* Top10 (Invitrogen) was transformed with the ligation mixture using electroporation using the *E. coli* gene pulser (BioRad). After phenotypic expression for 2 hours in SOC medium the culture was spread onto selective LB medium (Sambrook) containing 50 µg of ampicillin per ml. Transformed colonies were identified using PCR amplification and a positive clone designated pPK727 was further tested by restriction enzyme analysis. The functionality of the PCR amplified *RelE* gene was tested in *E. coli* by cloning the *NcoI-EcoRI* fragment from pPK727 into the *E. coli* expression vector pUHE24. Induction with IPTG led to cell killing in *E. coli*.

4. Yeast transformation

S. cerevisiae was grown overnight (ON) in YDP medium (1% yeast extract; 2% Bacto peptone; 2% glucose). For a single transformation, cells from 1 ml ON culture were spun down (5,000 rpm for 30 sec using an Eppendorf minicentrifuge) and washed twice in sterile water. Cells were resuspended in 200 µl lithium acetate buffer (10 mM Tris-HCl pH 7.6 with 100 mM LiOAc, 1 mM EDTA). After incubation for 15 min. at 25°C with agitation two transformations were made adding 20 µl carrier DNA (10 mg/ml salmon sperm DNA, sonicated and heat denatured) and 100 ng of the plasmids pPK727 and pYES2 (vector control), respectively. A volume of 1.2 ml 40% PEG 4,000 in 0.1 M lithium acetate buffer was added to each transformation mixture. The transformation mixtures were incubated in a 25°C incubator for 30 min before transferring to a 42°C water bath for 15 min. Cells were spun down (5,000 rpm for 30 sec.) and washed once with sterile water before plating on Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto yeast nitrogen base; 0.1% amino acids (without uracil); 2% agar).

After three days growth at 30°C single colonies were picked and streaked onto plates with Uracil drop-out medium. After two days at 30°C cells were transferred to induc-

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tion medium (Uracil drop-out medium with 2% galactose as the sole carbon source) by replica-plating.

Single colonies of *S. cerevisiae* containing pPK727 and pYES2 were transferred to liquid Uracil drop-out medium (1% Bernstein acid; 0.1% NaOH; 2% glucose; 0.67% Bacto-yeast nitrogen base; 0.1% amino acids (without uracil)) and incubated ON. To compensate for difference in cell density, a volume of 50 μ l per OD₅₄₀ (optical density at 540 nm) was used to inoculate 50 ml of liquid Uracil drop-out medium with either glucose or galactose as sole carbon source, respectively. The four flasks with *S. cerevisiae* (pPK727) and *S. cerevisiae* (pYES2) in Uracil drop-out medium with or without galactose were incubated at 30°C with moderate shaking (200 rpm). To monitor growth, samples were taken at different time point and OD₅₄₀ was determined. Samples were taken in duplicates and the average OD₅₄₀ calculated and plotted against time of sampling

5. Results.

All colonies containing either the plasmid pPK727 or the pYES2 control plasmid were able to grow on plates with glucose as carbon source. When transferred to plates with galactose as the sole carbon source leading to gene expression from the P-gal1 promoter only cells with the pYES2 control plasmid showed normal growth, whereas cells containing the pPK727 were strongly inhibited in growth.

In liquid media, the yeast cells in which the *relE* gene was induced showed a remarked growth inhibition when compared to the uninduced control and to the controls with only the plasmid pYES2 without insert.

These results that are summarised in the below Table 16.1 clearly suggest that inhibition of cell growth in yeast cells is due to expression of the *relE* gene.

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Table 16.1. Growth of *Saccharomyces cerevisiae* transformed with pYES2 +/- *relE* gene

time (hours)	Plasmid + <i>relE</i>		plasmid (control)	
	Galactose	no galactose	galactose	no galactose
0	0	0	0	0
5,5	0,018	0,024	0,023	0,028
15,5	0,013	0,186	0,055	0,176
23	0,190	1,195	0,286	0,963
28	0,010	1,480	0,816	1,649
29	0,035	3,930	1,660	2,650
65	0,990	3,950	6,180	3,820

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CLAIMS

1. A method of conditionally controlling the survivability of a recombinant microbial cell population, the method comprising (i) providing in the cells of said population a
5 gene coding for a cytotoxic first kind of polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide (said genes collectively being designated herein as the *relE* gene family), said gene is expressible in the cells of the population and, operably linked to the gene, a regulatable regulatory DNA sequence and (ii)
10 cultivating the cell population under conditions where the *relE* gene or the gene coding for a functionally equivalent polypeptide is expressed, the expression leading to an at least partial killing of the cell population.
2. A method according to claim 1 wherein the gene coding for the functionally
15 equivalent polypeptide is derived from a Gram-negative bacterium.
3. A method according to claim 2 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp.,
20 *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.
4. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.
- 25 5. A method according to claim 4 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.
6. A method according to claim 5 wherein the Gram-positive bacterium is *Bacillus*
30 *thuringiensis*.
7. A method according to claim 1 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

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8. A method according to claim 1 wherein the *relE* family gene is present on the chromosome of the cells.

9. A method according to claim 8 wherein the cells of the population do not contain a
5 gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the RelE polypeptide or the functional equivalent hereof.

10. A method according to claim 1 wherein the gene coding for the RelE polypeptide or the functional equivalent hereof is present on an extrachromosomal replicon.

10

11. A method according to claim 1 wherein the regulatory DNA sequence regulates the expression of the gene coding for the RelE polypeptide or the functionally equivalent polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or absence of a chemical compound in the cultiva-
15 tion medium.

12. A method according to claim 11 wherein the promoter is inducible by a chemical compound.

20 13. A method according to claim 11 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

25 14. A method according to claim 1 wherein the cells of the population comprises a gene coding for a second kind of polypeptide that is capable of binding to the *relE* polypeptide or the functional equivalent, said binding resulting in that the toxic effect of the RelE polypeptide or the functional equivalent is at least partially counteracted.

30 15. A method according to claim 14 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for the second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

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16. A method according to claim 14 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

17. A method according to claim 16 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

18. A method according to claim 1 wherein the expression of the *relE* gene family gene is stochastically regulated.

10

19. A method according to claim 18 wherein the stochastical regulation is effected by operably linking the *relE* gene family gene to a regulatory sequence that comprises an invertible promoter.

20. A method according to claim 18 wherein the stochastical regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

21. A method according to claim 1 wherein the cells contain a gene coding for a gene product of interest.

22. A method according to claim 21 wherein the gene product of interest is an immunologically active gene product.

23. A method according to claim 21 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

24. A method according to claim 21 wherein the gene product of interest is a pesticidally active product.

30

25. A method according to claim 24 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

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26. A method of confining an extrachromosomal replicon to a microbial cell population, the method comprising the steps of

(i) isolating a microbial cell naturally containing a gene belonging to the *relE* gene family coding for a first kind of polypeptide that, when it is expressed in the cell, acts as a toxin for the cell or, if the cell does not naturally contain a gene belonging to the *relE* gene family, introducing such a gene into the cell,

(ii) introducing into the cell the extrachromosomal replicon to be confined, said replicon containing a gene coding for a second kind of polypeptide that, by binding to the first kind of polypeptide, acts as an antitoxin for said first kind of polypeptide,

(iii) cultivating the cell under conditions where the genes coding for the first and the second kind of polypeptides are expressed, whereby a daughter cell that does not receive a copy of the extrachromosomal replicon is killed by the first kind of polypeptide being expressed in the absence of expression of the second kind of polypeptide.

27. A method according to claim 26 wherein the cell population consists of cells that comprises a gene coding for a gene product of interest.

28. A method according to claim 27 wherein the gene product of interest is selected from the group consisting of an enzyme, an immunologically active polypeptide, a pesticidally active gene product and a pharmaceutically active gene product.

29. A method according to claim 26 wherein the replicon is a plasmid occurring in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-10 such as the range of 1-5.

30. A method according to claim 26 wherein the microbial cells belong to a Gram-negative bacterial species.

31. A method according to claim 30 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

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32. A method according to claim 26 wherein the microbial cells belong to a Gram-positive bacterial species.

33. A method of post-segregationally stabilizing a plasmid in a microbial host cell population, the method comprising the steps of

(i) recombinationally inserting into the plasmid (a) a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a functional equivalent thereof, said first kind of polypeptide having a toxin effect on the host cell and (b) a gene coding for a second kind of polypeptide that (1) is capable of acting as an antitoxin for first kind of polypeptide and (2) is capable of being degraded in the host cell at a higher rate than that at which the first kind of polypeptide is degraded,

15 (ii) cultivating the cell population under conditions where the genes coding for the first kind and second kind of polypeptides are expressed,

whereby a daughter cell that does not receive at least one copy of the plasmid is killed as a result of the faster degradation of the second kind of polypeptide.

34. A method according to claim 33 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

35. A method according to claim 34 wherein the gene coding for the functionally
25 equivalent polypeptide is derived from a Gram-negative bacterium selected from the
group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp.,
Pseudomonadaceae spp., *Helicobacter* spp. and *Synechosystis* spp.

36. A method according to claim 33 wherein the gene coding for the functionally
30 equivalent polypeptide is derived from a Gram-positive bacterium.

37. A method according to claim 36 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

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38. A method according to claim 37 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

39. A method according to claim 33 wherein the gene coding for the functionally
5 equivalent polypeptide is derived from a species belonging to *Arhae*.

40. A method according to claim 33 wherein the second kind of polypeptide is selected from the group consisting of the relB polypeptide derived from *E. coli* K-12 and a functionally equivalent polypeptide.

10

41. A method according to claim 40 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

15 42. A method according to claim 33 wherein the plasmid contains a gene coding for a gene product of interest.

43. A method according to claim 42 wherein the gene product of interest is an immunologically active gene product.

20

44. A method according to claim 42 wherein the gene product of interest is one that is effective in degradation of an environmental pollutant.

25 45. A method according to claim 42 wherein the gene product of interest is a pesticidally active product.

46. A method according to claim 45 wherein the gene coding for the pesticidally active gene product is derived from *Bacillus thuringiensis*.

30 47. A method according to claim 33 wherein the microbial cells belong to a Gram-negative bacterial species.

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48. A method according to claim 47 wherein the bacterial species is selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.

5 49. A method according to claim 33 wherein the microbial cells belong to a Gram-positive bacterial species.

50. A method according to claim 33 wherein the plasmid is one that occurs in the microbial cells at a copy number which is in the range of 1-30 including the range of 1-

10 10 such as the range of 1-5.

51. A recombinant cell comprising a gene coding for a first kind of polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide and a gene coding for a functionally equivalent polypeptide or a variant or derivative hereof, said
15 first kind of polypeptide having a toxic effect on the cell, subject to the limitation that when the cell is *E. coli*, the gene coding for the first kind of polypeptide is not derived from *E. coli*.

52. A cell according to claim 51 wherein the cells belong to a bacterial species selected from the group consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp. and *Pseudomonadaceae* spp.
20

53. A cell according to claim 51 wherein the cells belong to a Gram-positive bacterial species.
25

54. A cell according to claim 51 wherein the gene coding for *E. coli* K-12 RelE polypeptide or the functional equivalent hereof is located on the chromosome.

55. A cell according to claim 51 wherein the gene coding for the *E. coli* K-12 RelE polypeptide or the functional equivalent hereof is located on an extrachromosomal replicon.
30

56. A cell according to claim 51 which comprises at least one gene coding for a gene product of interest.

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57. A cell according to claim 56 wherein the gene product of interest is an immunologically active gene product.

58. A cell according to claim 56 wherein the gene product of interest is one that is
5 effective in degradation of an environmental pollutant.

59. A cell according to claim 51 wherein the gene product of interest is a pesticidally active product.

10 60. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide whereby the toxic effect hereof is at least reduced.

61. A cell according to claim 51 comprising, operably linked to the gene coding for a
15 first kind of polypeptide, a regulatable regulatory DNA sequence.

62. A cell according to claim 61 wherein the regulatory DNA sequence regulates the expression of the gene coding for the first kind of polypeptide at the transcriptional level by means of a promoter, the function of which is regulated by the presence or
20 absence of a chemical compound in the cultivation medium.

63. A cell according to claim 62 wherein the promoter is inducible by a chemical compound.

25 64. A cell according to claim 62 wherein the promoter is suppressible by a first kind of chemical compound and inducible by a second kind of chemical compound whereby, when the first kind of compound is depleted from the medium, the promoter is induced by the second kind of compound.

30 65. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium.

66. A cell according to claim 65 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-negative bacterium selected from the group

FOOTNOTES

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consisting of *Enterobacteriaceae* spp., *Hemophilus* spp., *Vibrionaceae* spp., *Pseudomonadaceae* spp., *Helicobacter* spp. and *Synechosystis* spp.

67. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium.

68. A cell according to claim 67 wherein the gene coding for the functionally equivalent polypeptide is derived from a Gram-positive bacterium selected from the group consisting of lactic acid bacterial spp., *Bacillaceae* spp. and *Mycobacterium* spp.

69. A cell according to claim 68 wherein the Gram-positive bacterium is *Bacillus thuringiensis*.

70. A cell according to claim 51 wherein the gene coding for the functionally equivalent polypeptide is derived from a species belonging to *Arhae*.

71. A cell according to claim 51 which does not contain a gene coding for a second type of polypeptide that is capable of counteracting the cell toxic effect of the first kind of polypeptide.

72. A cell according to claim 51 which comprises a gene coding for a second kind of polypeptide that is capable of binding to the first kind of polypeptide, said binding resulting in that the toxic effect of the first kind of polypeptide is at least partially counteracted.

73. A cell according to claim 72 wherein the gene coding for the second kind of polypeptide is operably linked to a regulatable regulatory DNA sequence, permitting that the gene coding for said second kind of polypeptide is suppressed under conditions where the gene coding for the RelE polypeptide or the functional equivalent is expressed.

74. A cell according to claim 72 wherein the second kind of polypeptide is the RelB polypeptide derived from *E. coli* K-12 or a functionally equivalent polypeptide.

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75. A cell according to claim 74 wherein the functionally equivalent second kind of polypeptide is derived from the group consisting of a Gram-positive bacterium, a Gram-negative bacterium and *Archae* spp.

5 76. A cell according to claim 61 wherein the expression of the gene coding for the first kind of polypeptide is stochastically regulated.

77. A cell according to claim 76 wherein the stochastical regulation is effected by operably linking the gene coding for the first kind of polypeptide to a regulatory sequence
10 that comprises an invertible promoter.

78. A cell according to claim 76 wherein the stochastical regulation is effected by flanking at least part of the regulatory sequence by repeat sequences whereby the at least part of the regulatory sequence is recombinationally excised.

15 79. A composition comprising a cell according to any of claims 51-78.

80. A method of limiting the survival of a cell population in a first or a second environment, which method comprises

20 (i) transforming the cells of said population with a gene coding for a cytotoxic polypeptide, the gene is selected from the group consisting of the gene coding for the *E. coli* K-12 RelE polypeptide, the gene coding for the plasmid F CcdB polypeptide, the gene coding for the plasmid R1 PemK polypeptide, the gene coding for plasmid RP4
25 ParE polypeptide, the gene coding for the prophage P1 Doc polypeptide and a gene coding for a functionally equivalent polypeptide for anyone of said polypeptides, said gene is expressible in the cells of the population, and operably linked to the gene, a regulatory DNA sequence being regulatable by an environmental factor and which regulates the expression of said gene, and

30 (ii) cultivating the cell population under environmental conditions where the gene coding for the cytotoxic polypeptide is expressed, the expression leading to an at least partial killing of the cell population.

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81. A method according to claim 80 wherein the survival of the cell population is limited in a first environment in which the gene is expressed, said cell population thereby being contained in said first environment.

5 82. A method according to claim 80 wherein the survival of the cell population is not limited when present in a first environment, which first environment could change to a second environment physically and/or chemically distinct from the first environment, in which first environment the gene whose expression results in the formation of a cytotoxically active polypeptide is not expressed, but the survival of which cell population
10 is limited when transferred to a second environment or when present in a physically and/or chemically changed first environment, where the gene is expressed.

83. A method according to claim 80 wherein the survival of a cell population is being limited by providing in the cells a gene coding for a cytotoxic polypeptide which is operably linked to a nucleotide sequence encoding an antitoxin repressor substance
15 which can undergo a decay when said cells are released to the outer environment to an extent whereby the repressor substance is converted to a non-functional form, whereby as a result of said decay, the function of the cells of the population will be gradually limited.

20

84. A method of containing an extrachromosomal recombinant replicon to a first kind of cell, where said replicon is naturally transferable to a second kind of cell, which method comprises providing on the recombinant extrachromosomal replicon a gene whose expression results in the formation of a cytotoxic polypeptide selected from the
25 group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally equivalent polypeptide for anyone of said polypeptides to an extent whereby the function of the cell is being limited, said first kind of cells having or being modified to have a chromosomal replicon comprising a regulatory
30 nucleotide sequence the gene product of which inhibits the expression of said gene or the cell function-limiting effect of the polypeptide and thereby protects said first kind of cells, said regulatory gene being lacking in said second kind of cell, whereby, if a cell of the second kind receives said extrachromosomal recombinant replicon said gene is expressed and has a function-limiting effect on said second kind of cell.

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85. A method according to claim 84 wherein the gene product which inhibits the expression of the expression of the gene coding for the polypeptide or the cell function-limiting effect of the polypeptide is selected from the *E. coli* relB polypeptide, the plasmid F CcdA polypeptide, the plasmid R1 PemI polypeptide, the plasmid RP4 ParD
5 polypeptide, the prophage P1 Phd polypeptide and a functionally equivalent polypeptide of anyone of such polypeptides.

86. A method of stochastically limiting in an environment the survival of a cell population, the method comprising transforming the cells thereof with a recombinant replicon
10 containing a regulatably expressible gene which, when expressed in a cell encodes a cytotoxic polypeptide selected from the group consisting of the *E. coli* K-12 RelE polypeptide, the plasmid F CcdB polypeptide, the plasmid R1 PemK polypeptide, the plasmid RP4 ParE polypeptide, the prophage P1 Doc polypeptide and a functionally
15 equivalent polypeptide for anyone of said polypeptides, the expression of said gene leading to formation of the polypeptide to an extent whereby the function of the cells is being limited, the expression of said gene is stochastically induced as a result of recombinational excision of an excisable negatively functioning regulatory nucleotide sequence which, while present in the cells, inhibits expression of the gene coding for the
20 polypeptide, said negatively functioning regulatory nucleotide sequence being contained in the recombinant replicon or in an other recombinant replicon present in cells of the population containing the replicon.

87. A cell according to claim 51 that is an animal cell.

25 88. A cell according to claim 87 that is selected from the group consisting of a mammal cell, a human cell and an insect cell.

89. A method according to any one of claims 1, 26, 32, 80, 84 or 86 wherein the first kind of polypeptide inhibits translation.

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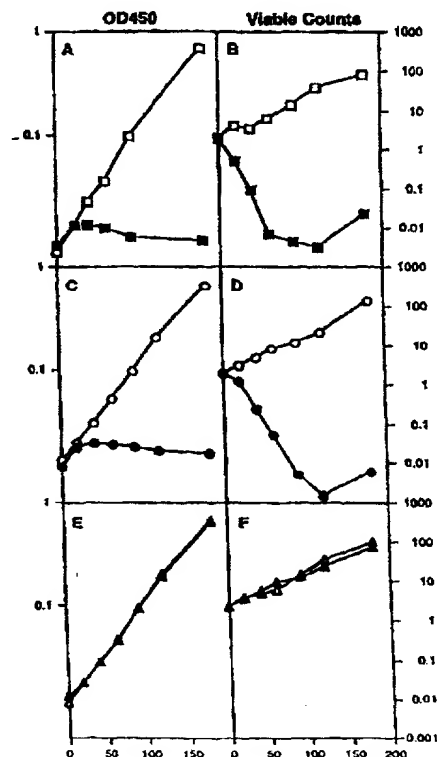
Without international search report and to be republished upon receipt of that report.

With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT

(57) Abstract

Method of conditionally controlling the survivability of a recombinant cell population and of containing such cells to an environment or containing replicons to a host cell is based on the use of proteic killer systems including the *E. coli relBE* locus and similar systems found in Gram-negative and Gram-positive bacteria and *Archae*. Such system are generally based on a cytotoxin polypeptide and an antitoxin or antidote polypeptide that in contrast to the cytotoxin is degradable by proteases. The recombinant cells are useful as vaccines, pollutant degrading organisms or as biological pest control organisms e.g. expressing *B. thuringiensis* crystalline proteins.



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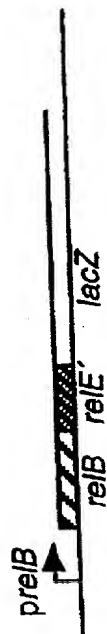
Plasmids	relBE derived fragments	β -galactosidase activity	Fold of repression
pKG4001		1250	1
pKG4002		28	45

Fig. 1

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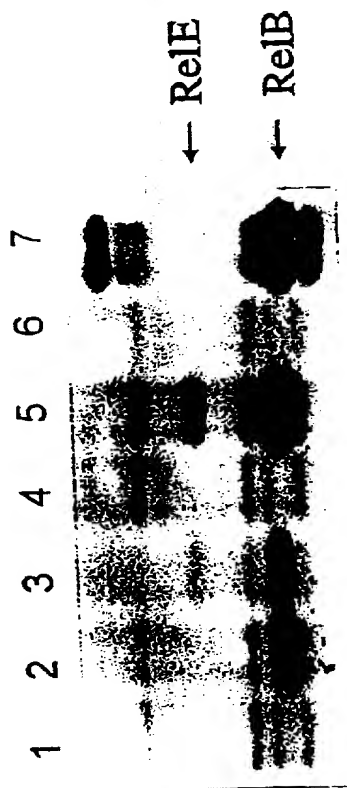


Fig. 2

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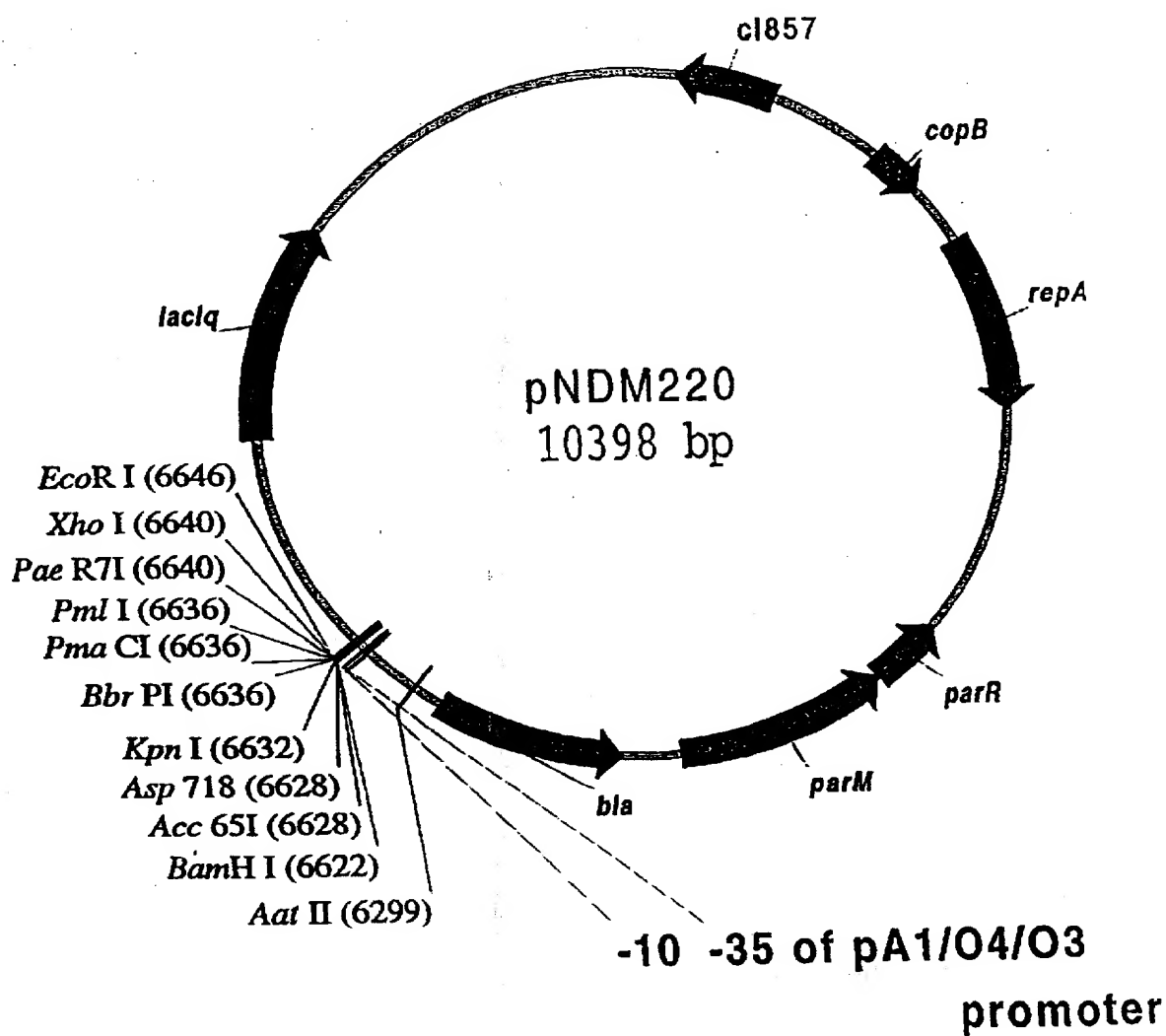


Fig. 3

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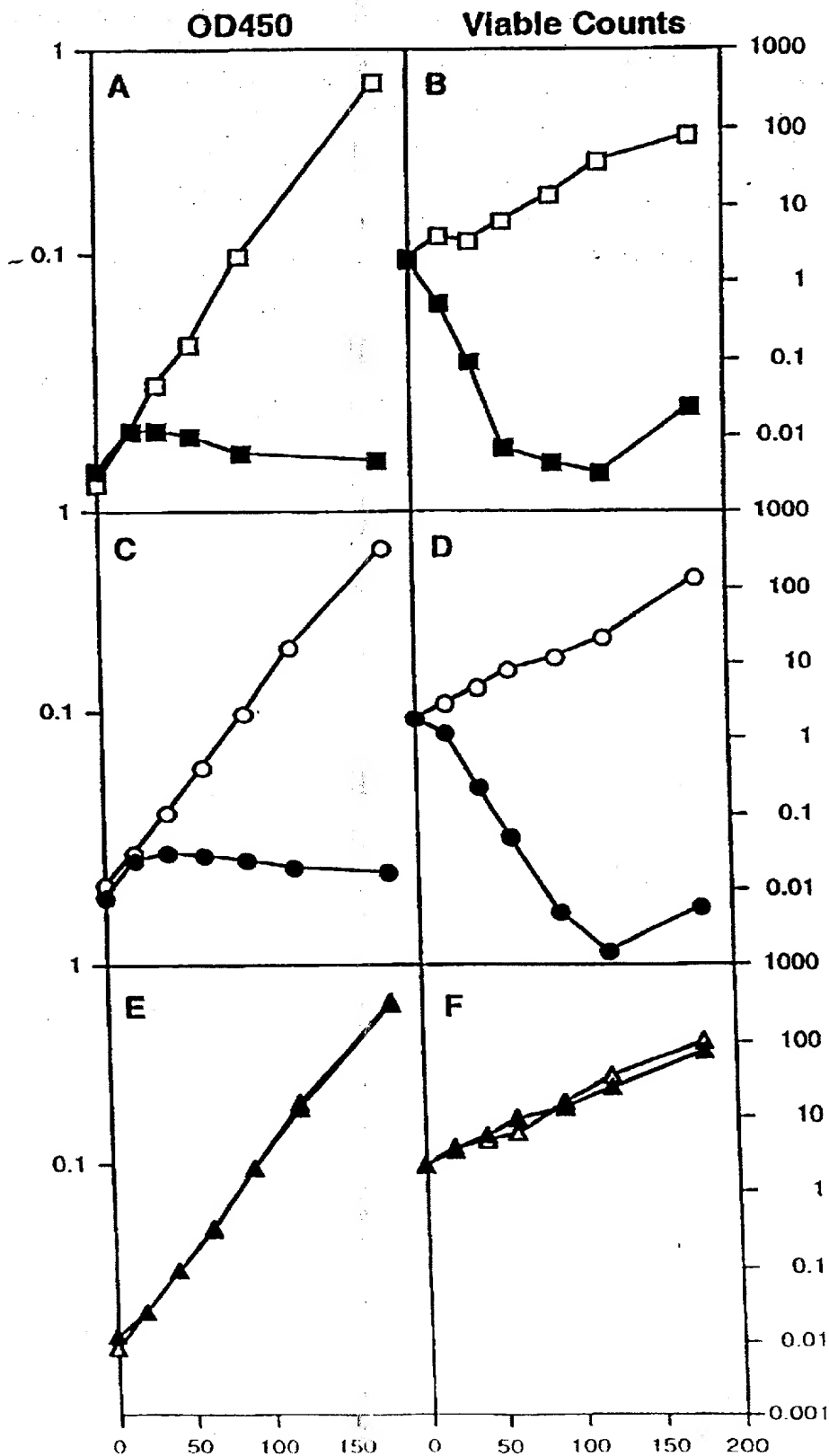


Fig. 4

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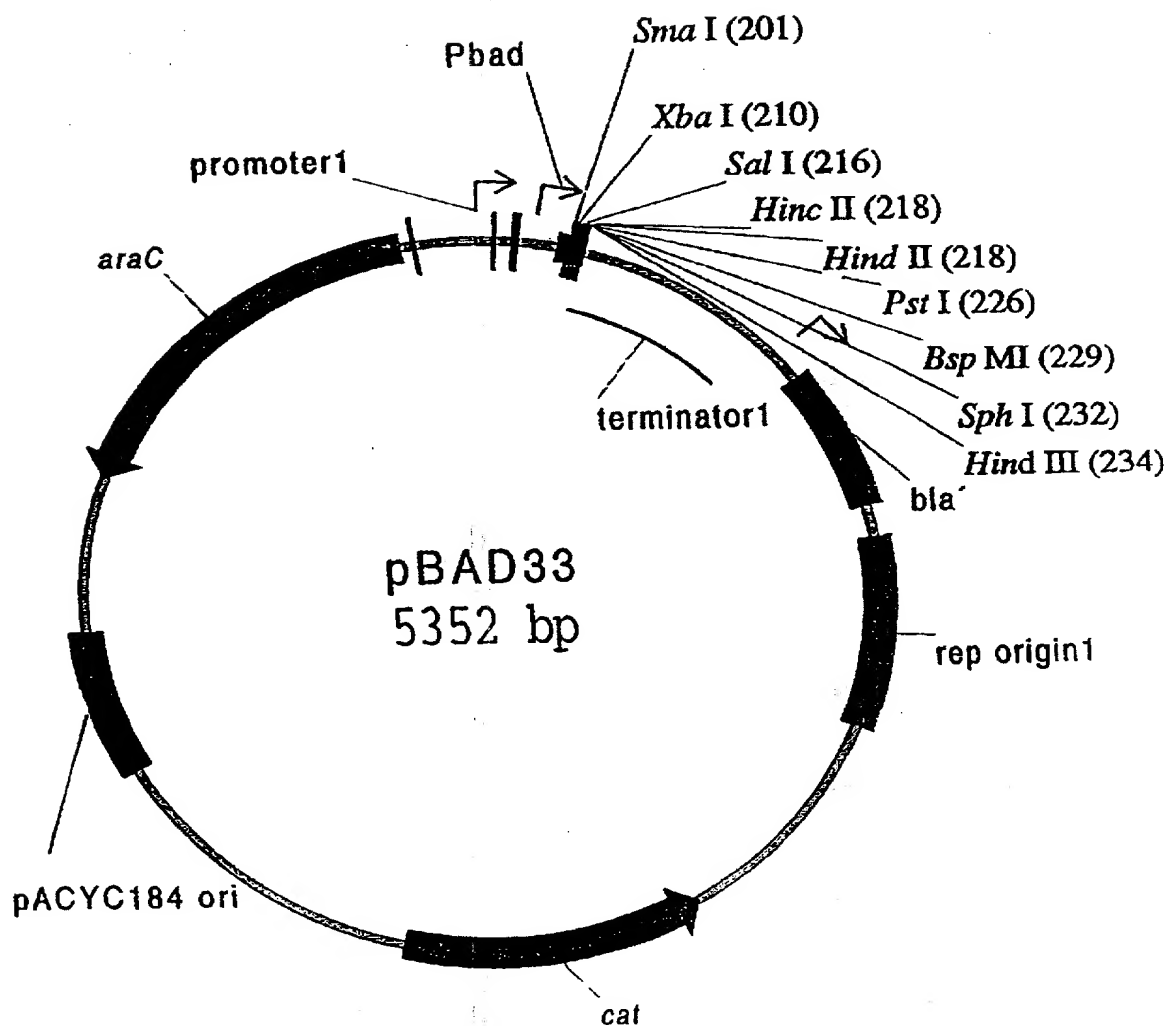


Fig. 5

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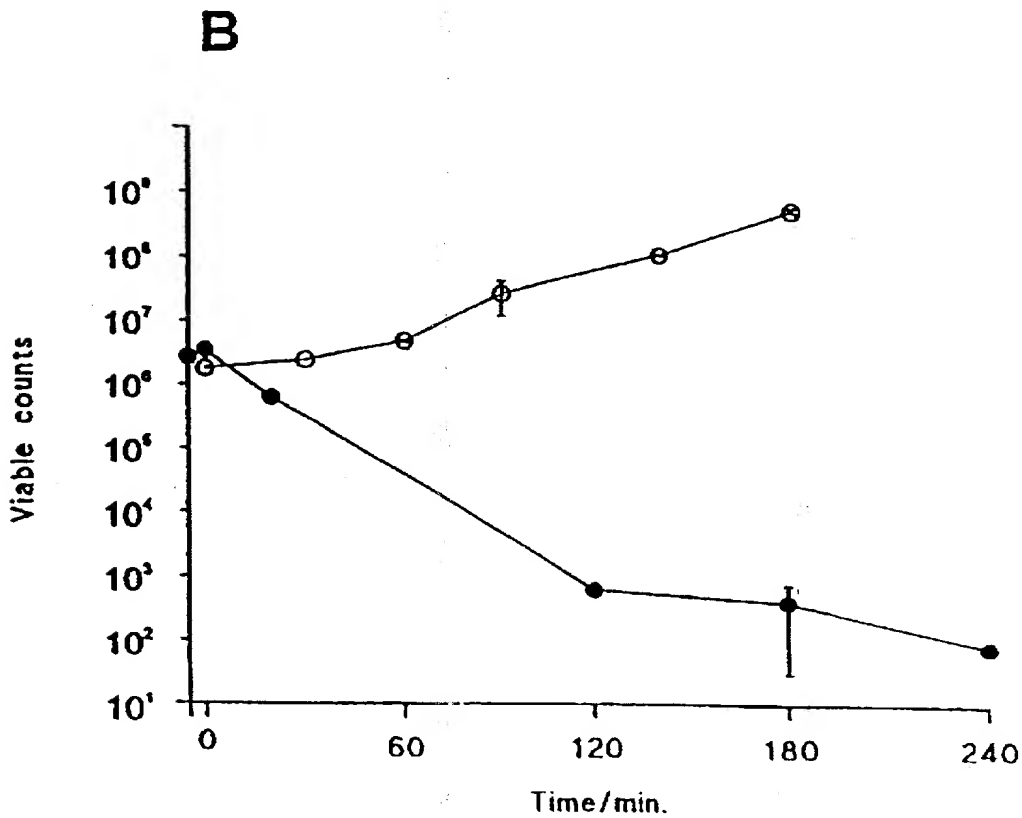
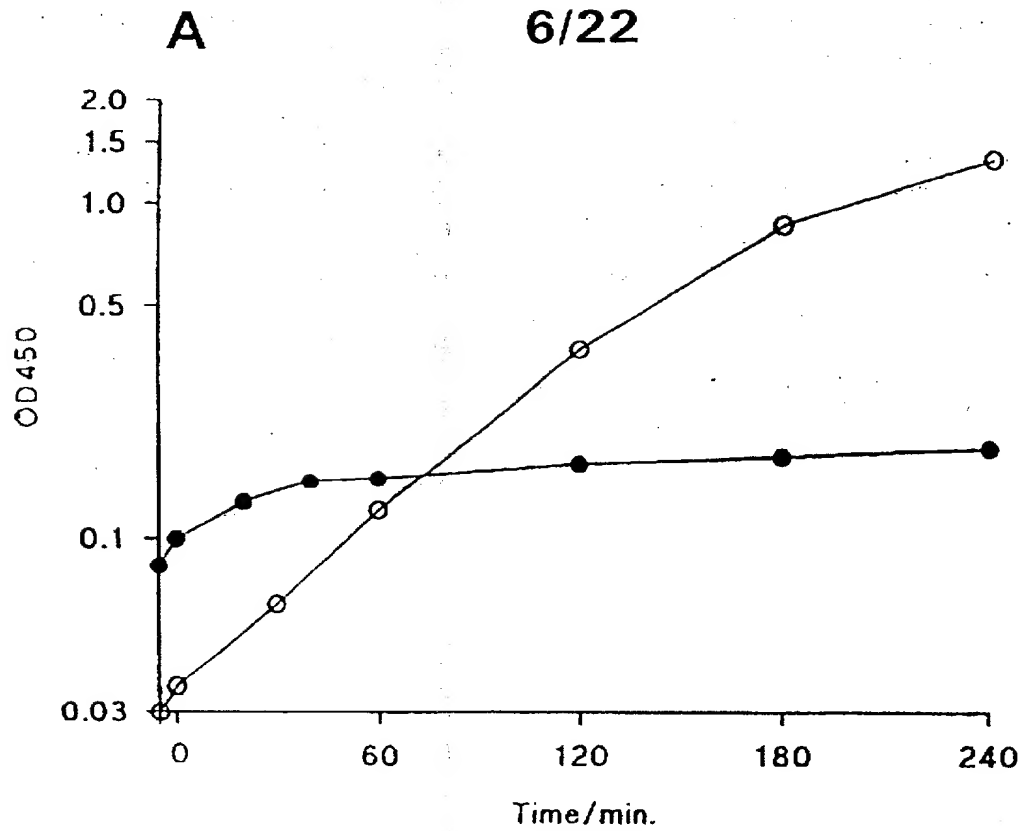


Fig. 6A

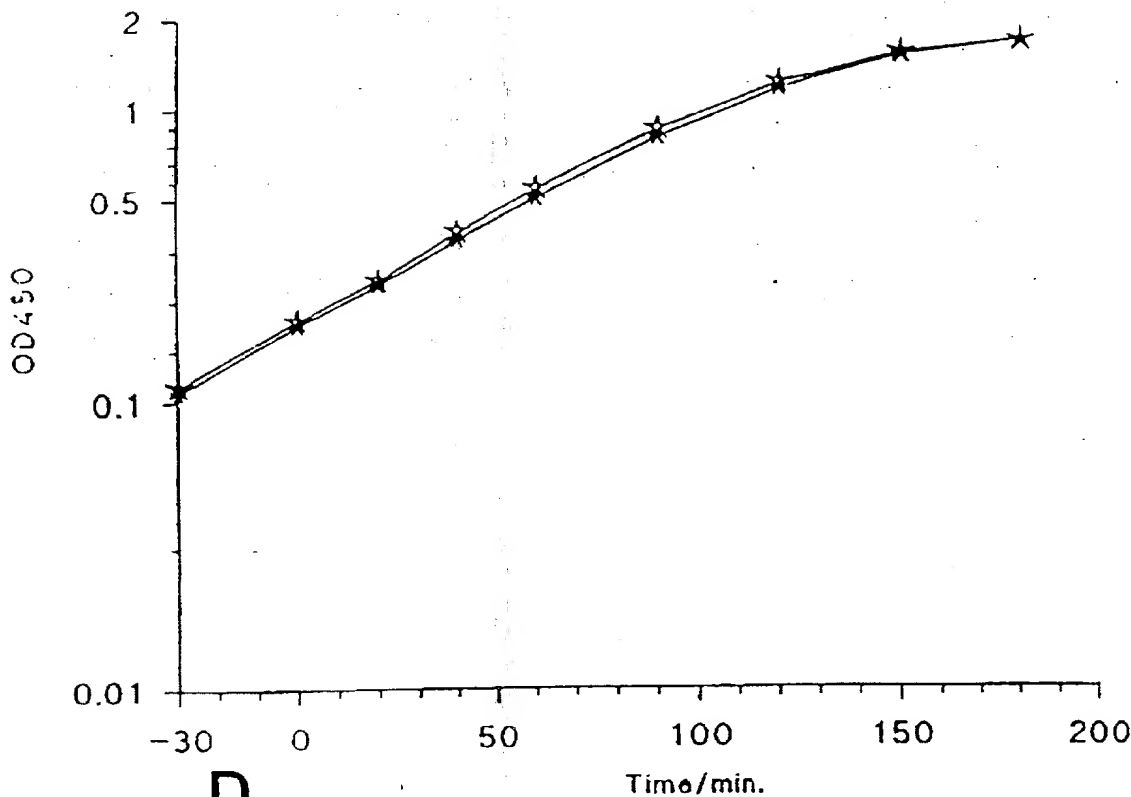
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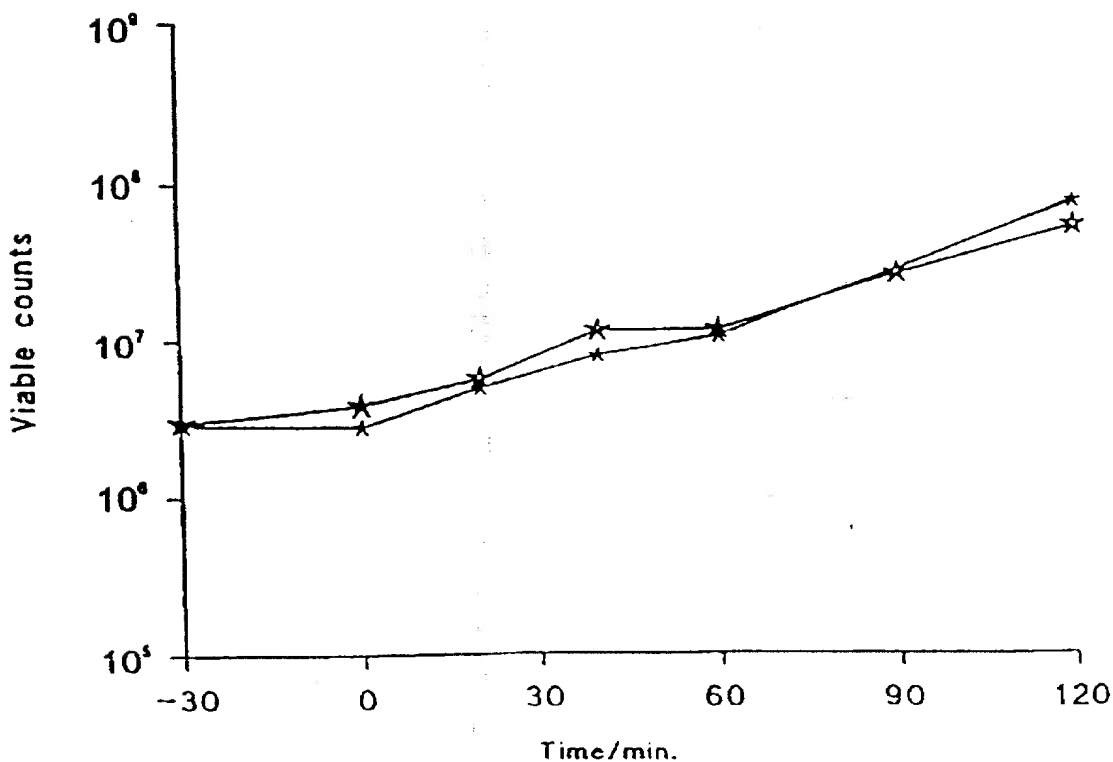


Fig. 6B

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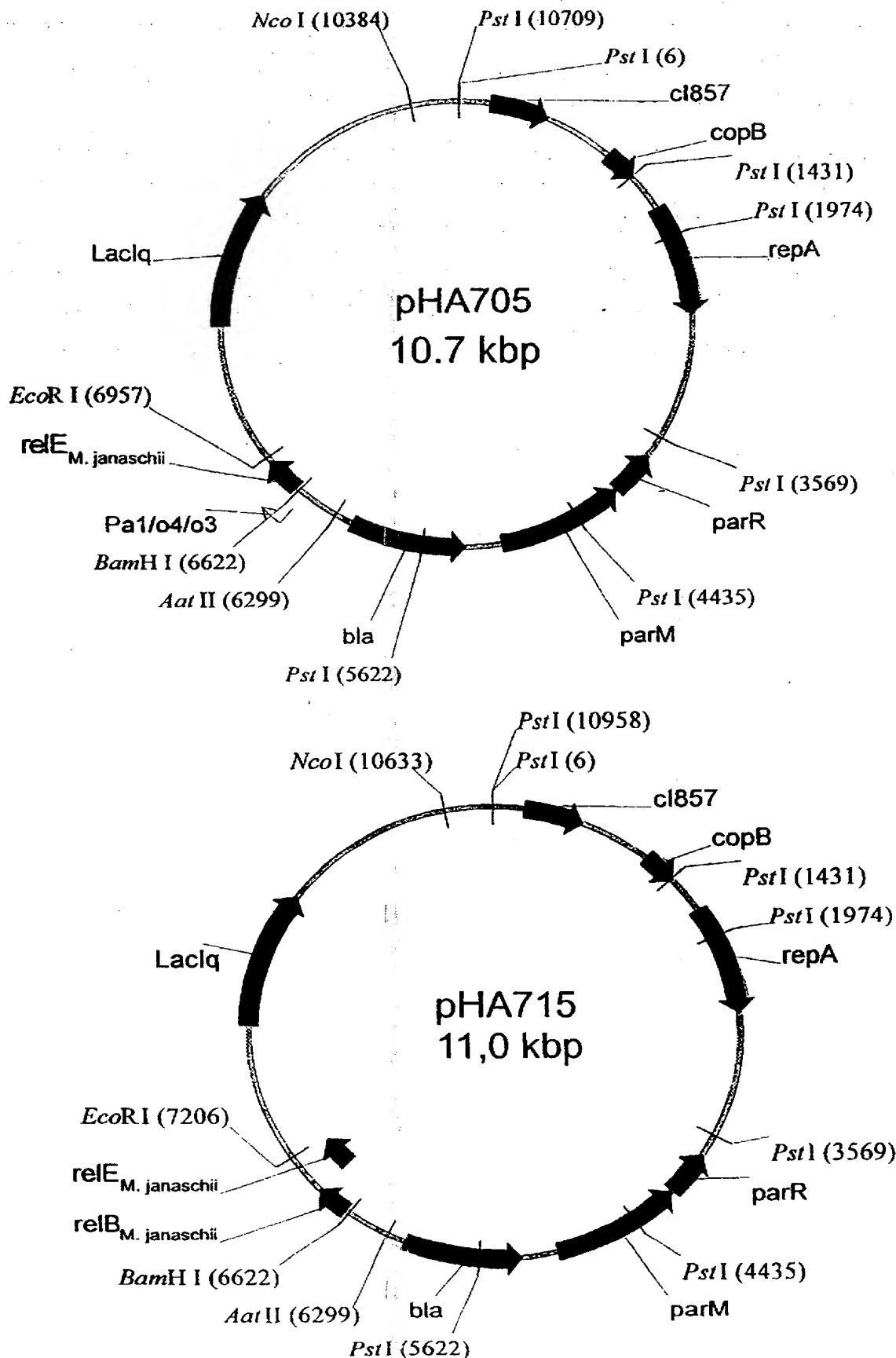


Fig. 7

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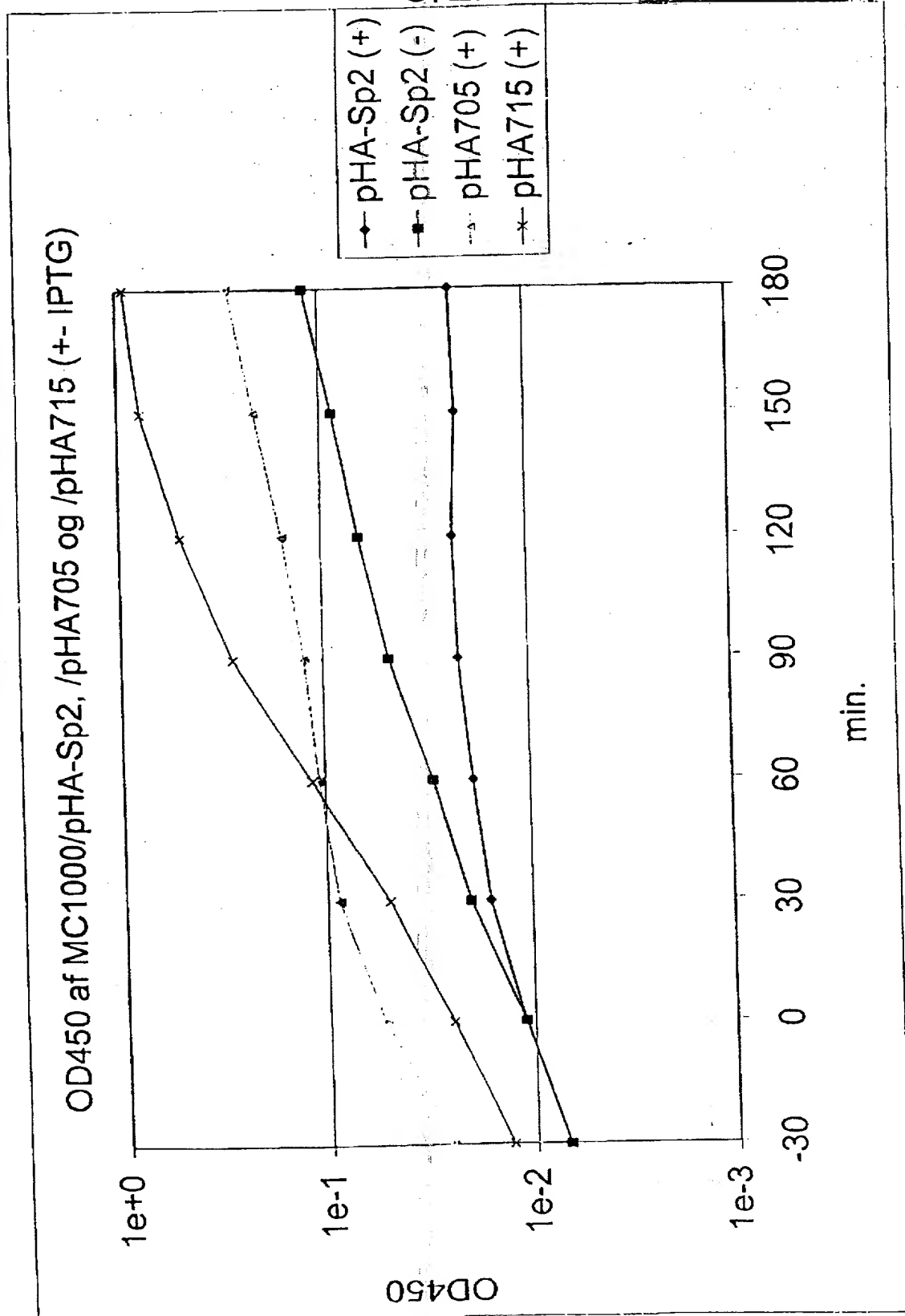


Fig. 8

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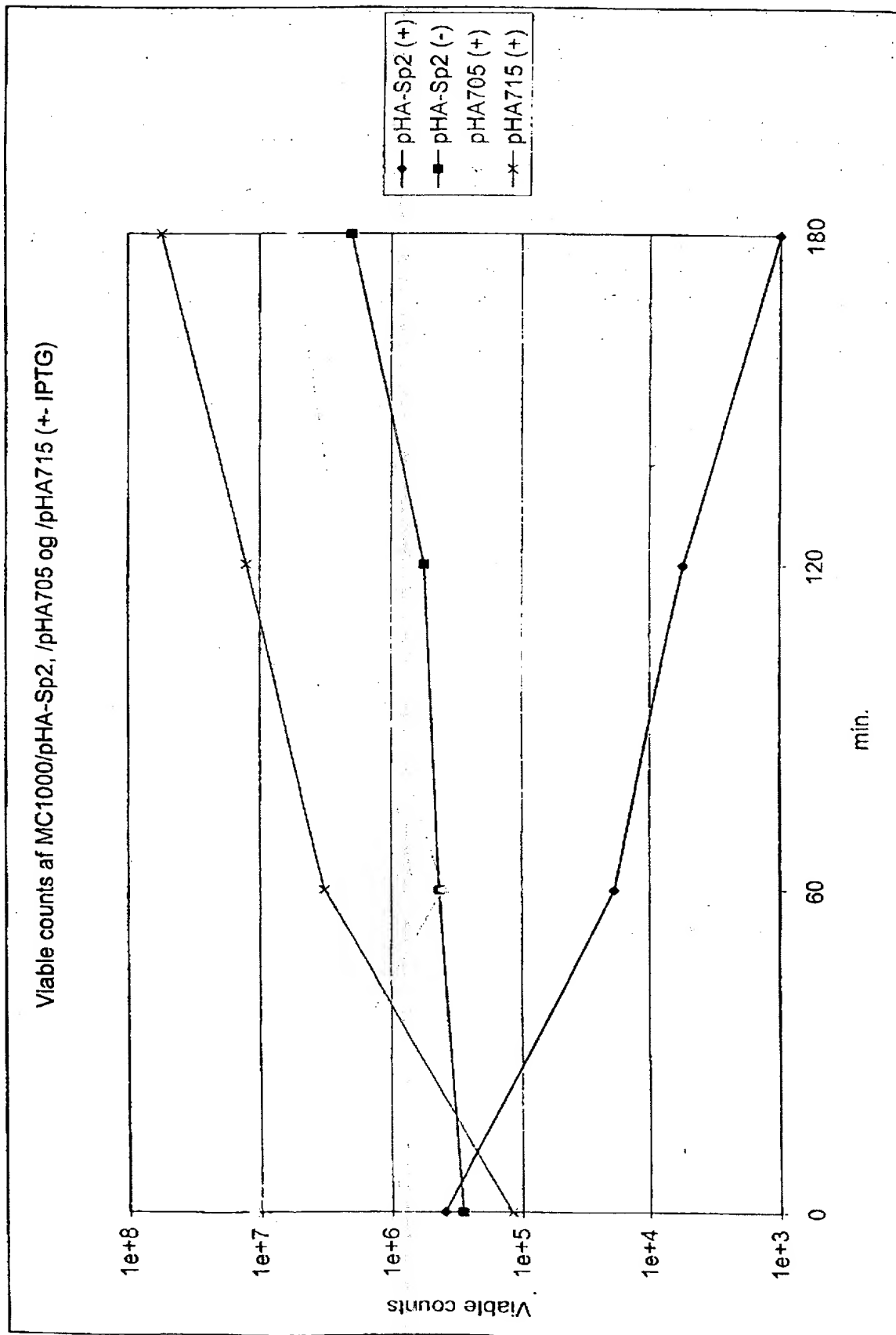


Fig. 9

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DNA sequence of the *relB_{sp2}* locus of *S. pneumoniae* (reverse complement of part of contig 80 in the tigr database)

1 ACCCATGAGC ATTCAGATCA TATCCATGGA GTAGGCGTTT TGGCTCGCAA
 51 GTATGGTATG GATCTTTATG CCAATGAAAA GACCTGGCAA GCTATGGAAA
 101 ATAGTAAATA TCTTGGCAAG GTGGATTCTT CGCAAAAGCA TATTTTGTAA
 151 ATGGGTAAAA CCAAAACCTT TGGAGATATC GACATCGAGA GTTTTGGTGT
 201 AAGCCATGAT GCAGTCGCAC CGCAGTTCTA TCGCTTTATG AAGGATGATA
 251 AGAGTTTTGT CCTCTTGACA GATACAGGTT ATGTCAGTGA CCGTATGGCG
 301 GGCATTGTCTG AAAATGCGGA TGGTTATCTT ATCGAGGCCA ACCATGATGT
 351 AGAGATTTTG CGATCAGGTT CTTACGCTTG GCGACTCAAA CAACGAATCC
 401 TATCTGACCT TGGTCACCTT TCTAACGAGG ACGGTGCTGA AGCTATGATT
 451 CGGACGCTAG GAAATCGTAC TAAGAAGATT TACCTTGGGC ATTTATCTAA
 501 GGAAAACAAT ATCAAGGAAC TGGCTCATAT GACCATGGTC AATCAGCTGG
 551 CTCAAGCTGA TCTGGGAGTC GGAGTAGACT TTAAGGTTTA TGATACCTCA
 601 CCAGATACCG CAACACCATT GACAGAGATA TAGAAAGAAC GCTGAGAAGG
 651 TGTTCCTTTT ATATTGACTG AACACCTAAA AAGTAATACA ATGGTGTTAC
 SD Start *relB_{sp2}*
 701 CATTAACAAA GGGAGCACAA AAGATGACTA CTATTACATT AAAAGTTTCT
 751 GAAGCTGATA AAACATTAT GAAAGCAATG GCTAAGTTTG AAGGAGTTTC
 801 CCTTTCGGAA CTTATTGCGA CCAAACTCT TGAAGCCCTA GAAGATGAAT
 851 ACGATGCTCG TGTGGCAGAT TTAGCCTATC AAGAGTATTT AGAAGACTTG
 SD
 901 GAAAAAGGAG TTGAACCCAT TACTTGGGAA GAAATGATGC ATGATTTAGG
 Start *relE_{sp2}*/End *relB_{sp2}*
 951 CTTGAAGGAT GAATAATTG TATAAATTAG TTCCAACAAG ACGTTTTATC
 1001 AAGCAATTGA AAAAATTGGA CCGTTATACG CAGAAGCTAA TTACAACTA
 1051 TTTACAAACC AATGTTTTGG AAGACCCAAG ACGACACGGA AAGGCTTTGG
 1101 TTGGTAATCG CGTTGGTCAA TGGCGCTATA GAATTGGTAA TTATCGAGTT
 1151 ATCGTACAAA TTGTAGATGA TGAATTAGTC GTTGCTACTC TAGAAGTTGG
 End *relE_{sp2}*
 1201 TCATCGGAGA GATATTTATT GAATTACTTT TTTCAAGTAA ATTTTCATTC
 1251 TTTAAAATAT GATATAATAG GGCTTACAAT ATAAGAGAGG TTGCTGATGA
 1301 CAATAGATAT TAGTGAAGAA AGTTTGGCTA AAGAATCGGC TGACTTATTA
 1351 AAAATTCTTT TAAAAGATCG AACGACAAAG AAATCAATTG TTTGGGCTAC
 1401 GCATTCTTAT GAATTGTTAG GAAAGGGATT TGCTCCAAGT GATCGCATCA
 1451 ATCCAAGTAA GGTTACTGGA AACTTTGCCA ACTTGATTCA GCCCAGATCT
 1501 GAAAAATCCA AATATGAACA AAAAGATCGG ACAAAAATAA GGGCAGAAGT

Protein sequences:

RelB_{sp2} (pHi=4.2):

mttitlkvse adktfmkama kfegvslsel irtktleale deydarvadl aygeyledle
 kgvepitwee mmhdlglkde

RelE_{sp2} (pHi=11.1):

mnlyklvpt rrfikqlkl drytqklitn ylgtnvledp rrhgkalvgn
 rvgqwryrig nyrvivqivd delvvatlev ghrrdiy

Fig. 10

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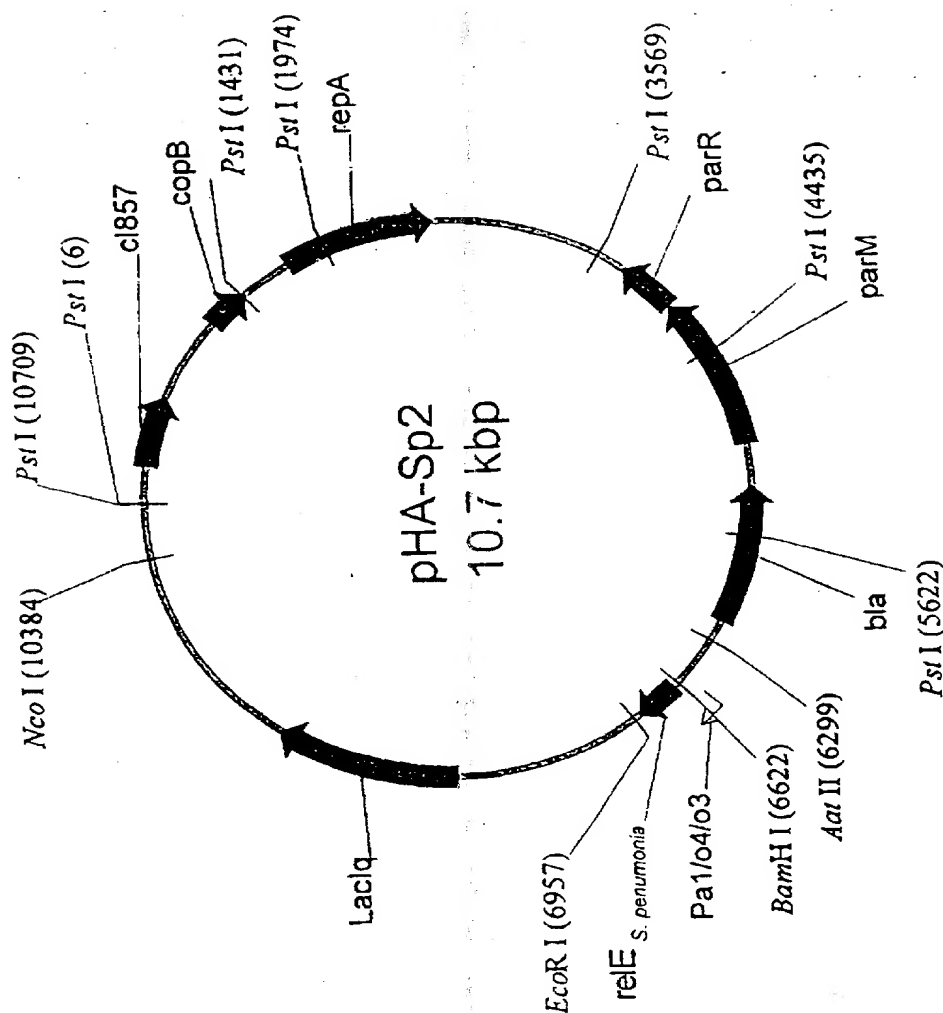


Fig. 11

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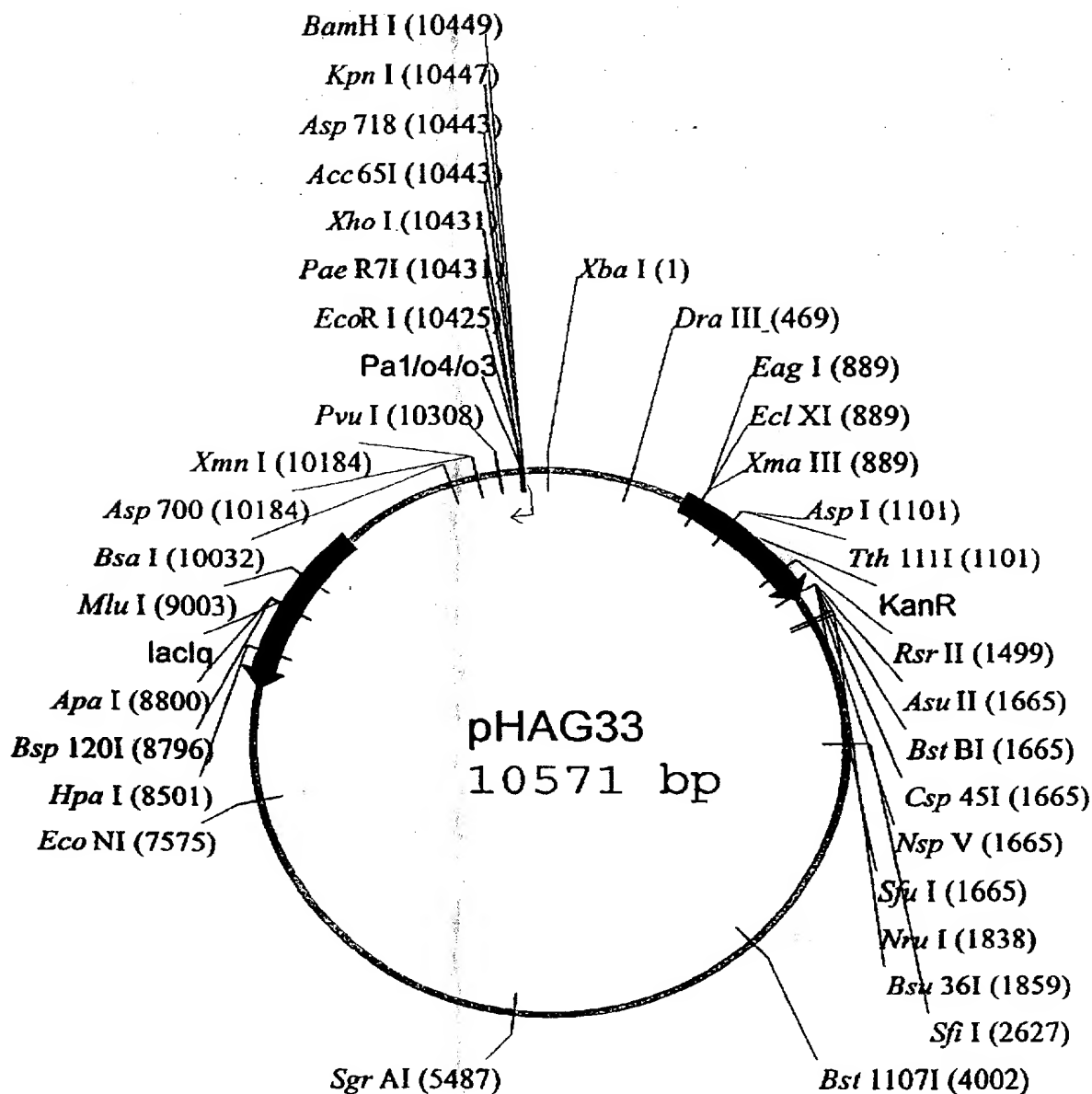


Fig. 12

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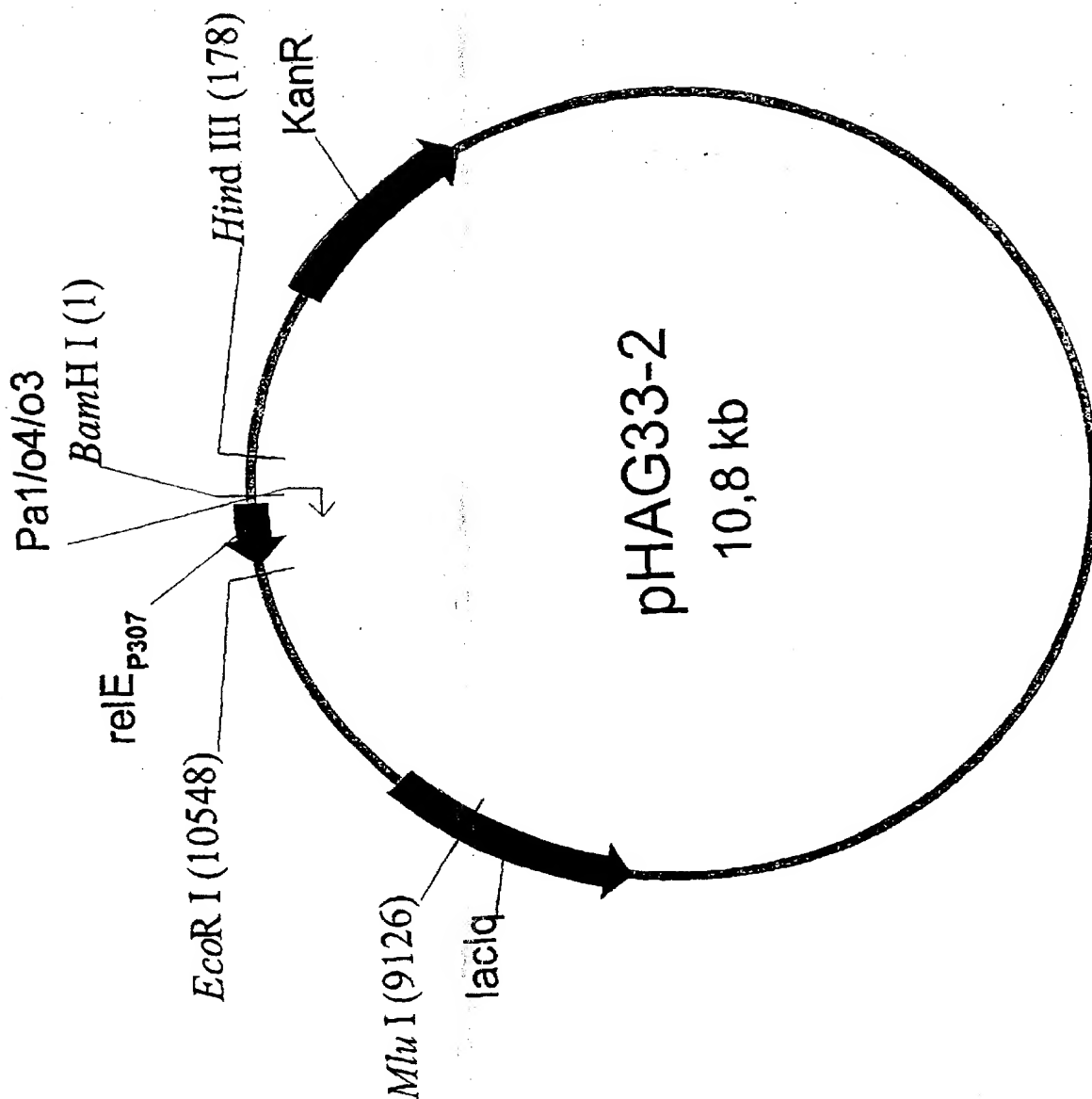


Fig. 13

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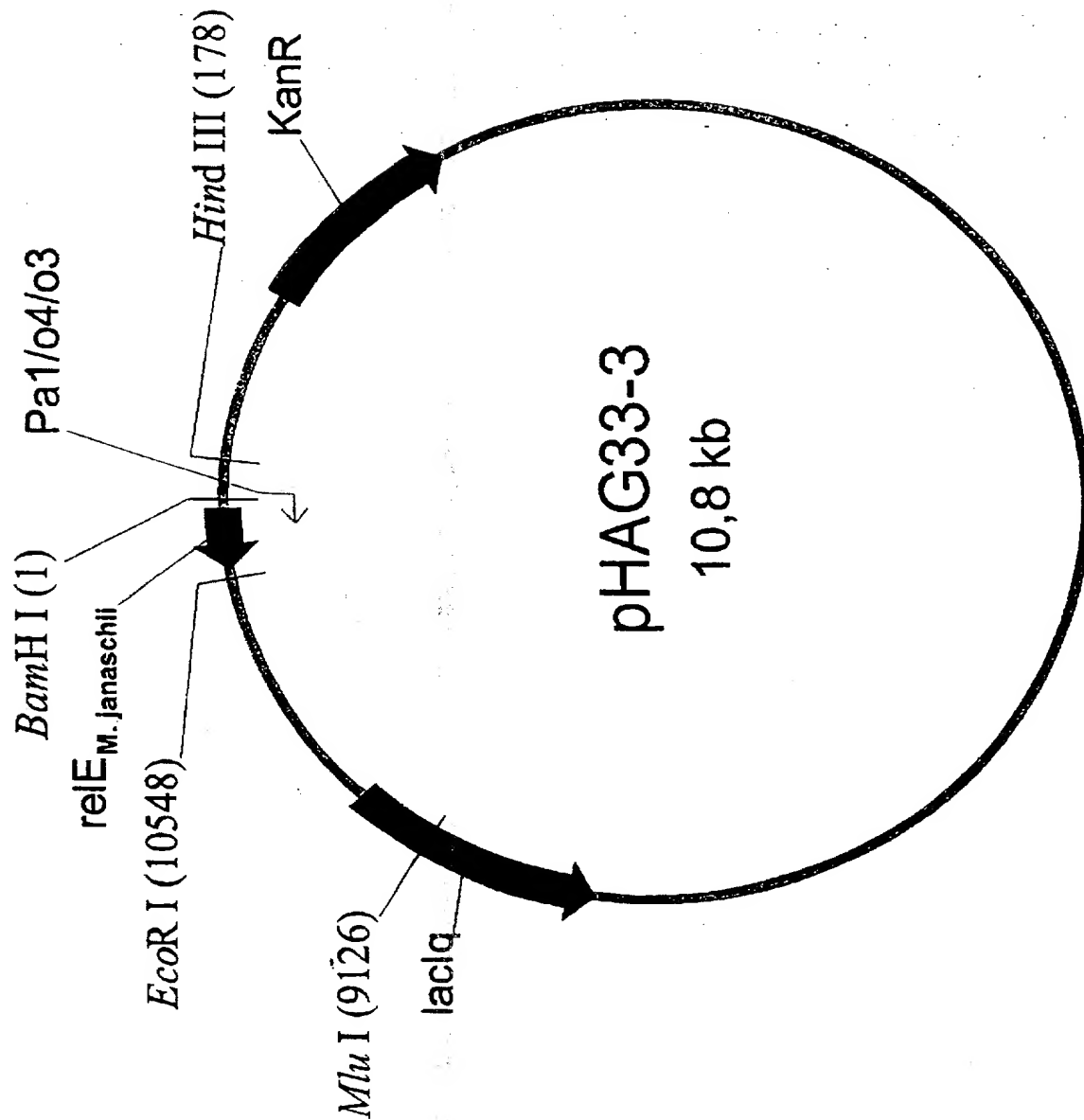


Fig. 14

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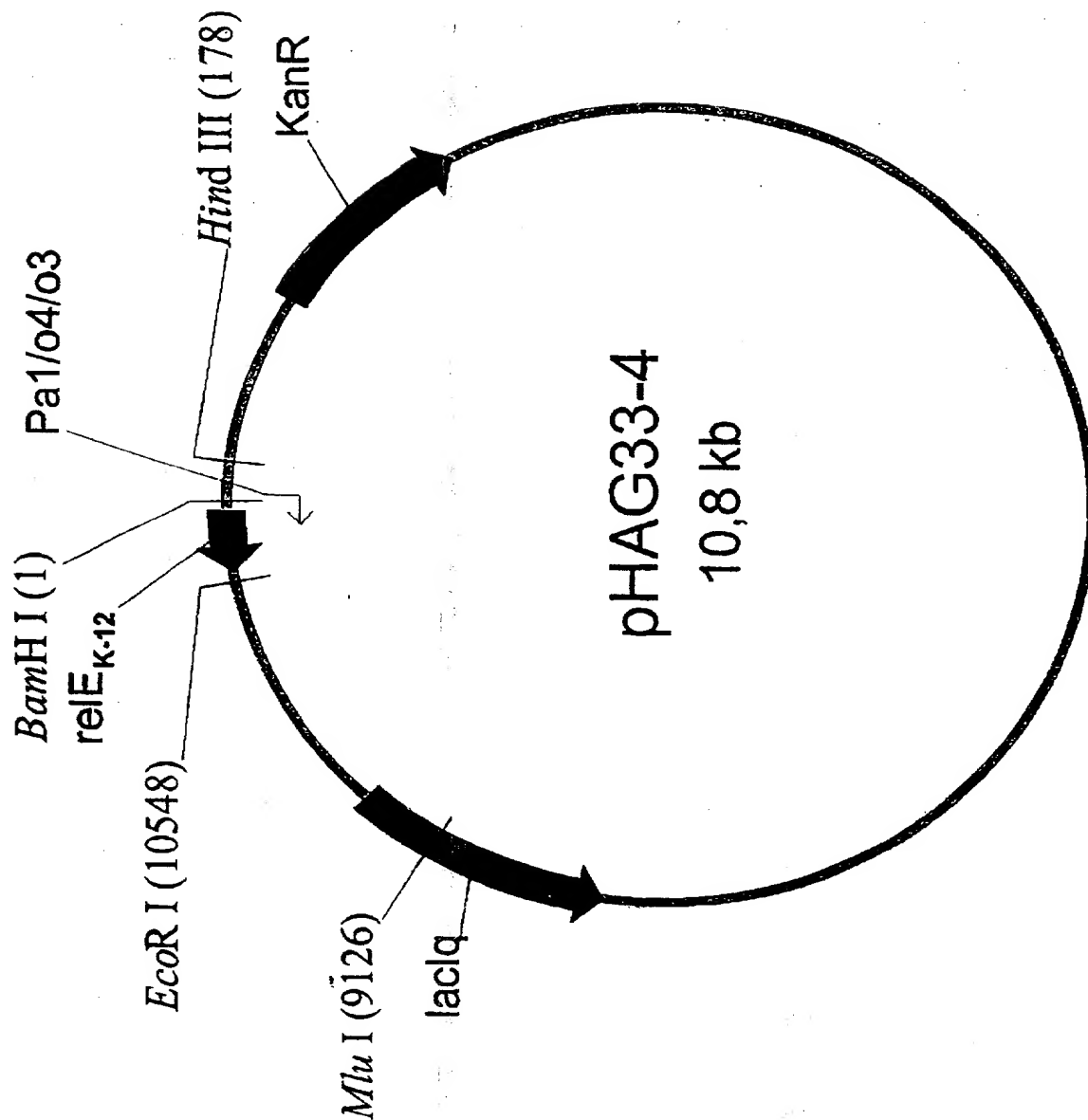


Fig. 15

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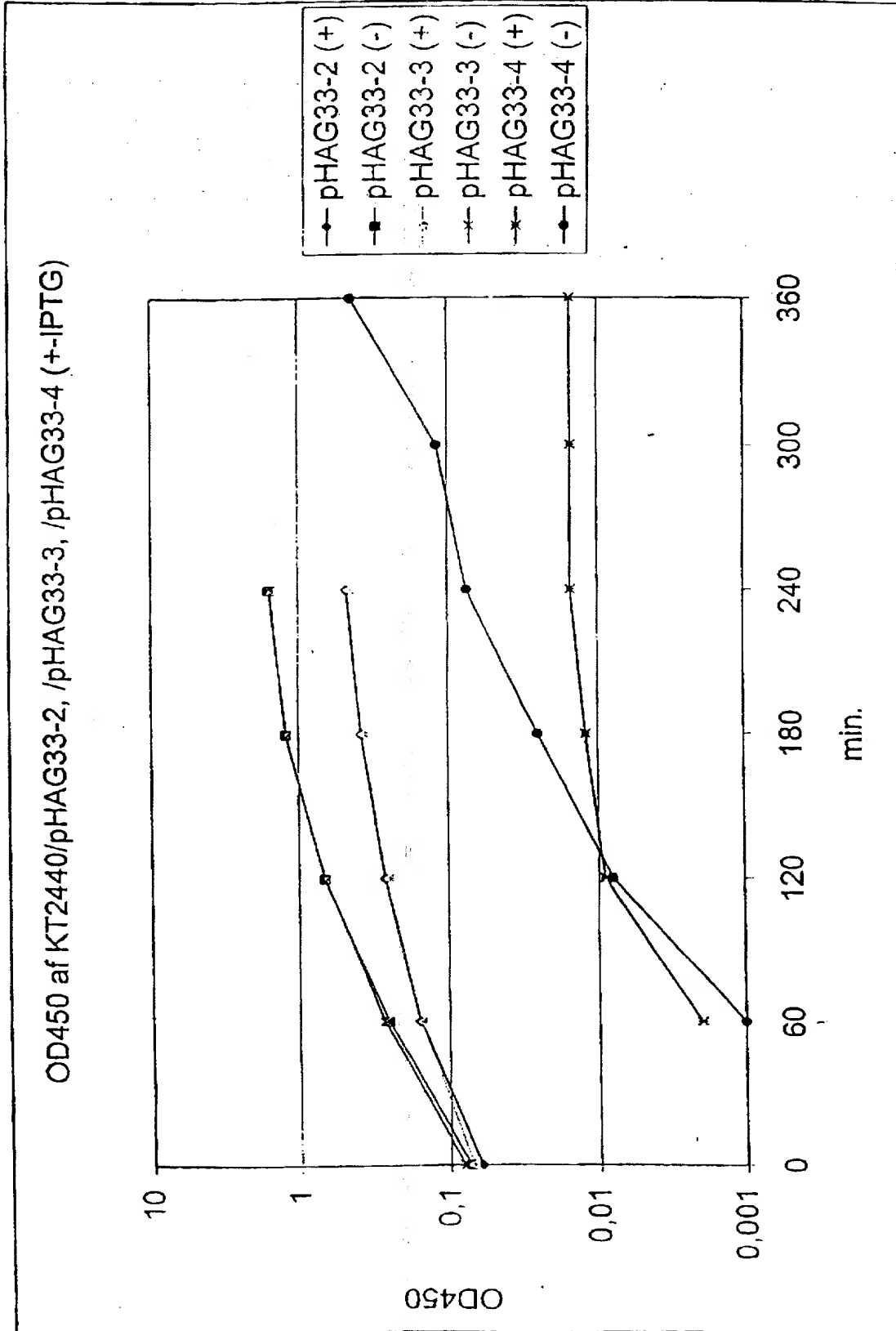


Fig. 16

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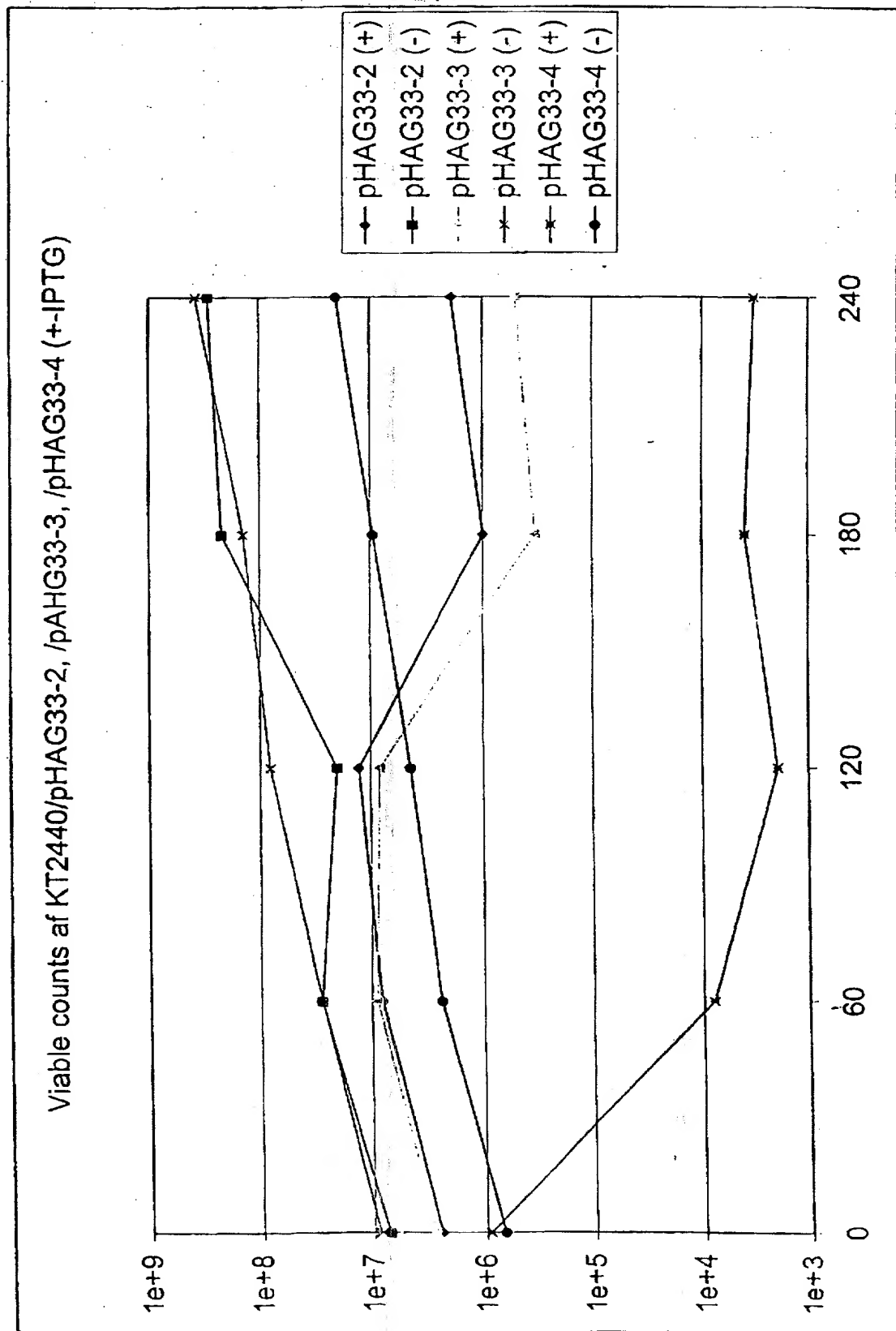


Fig. 17

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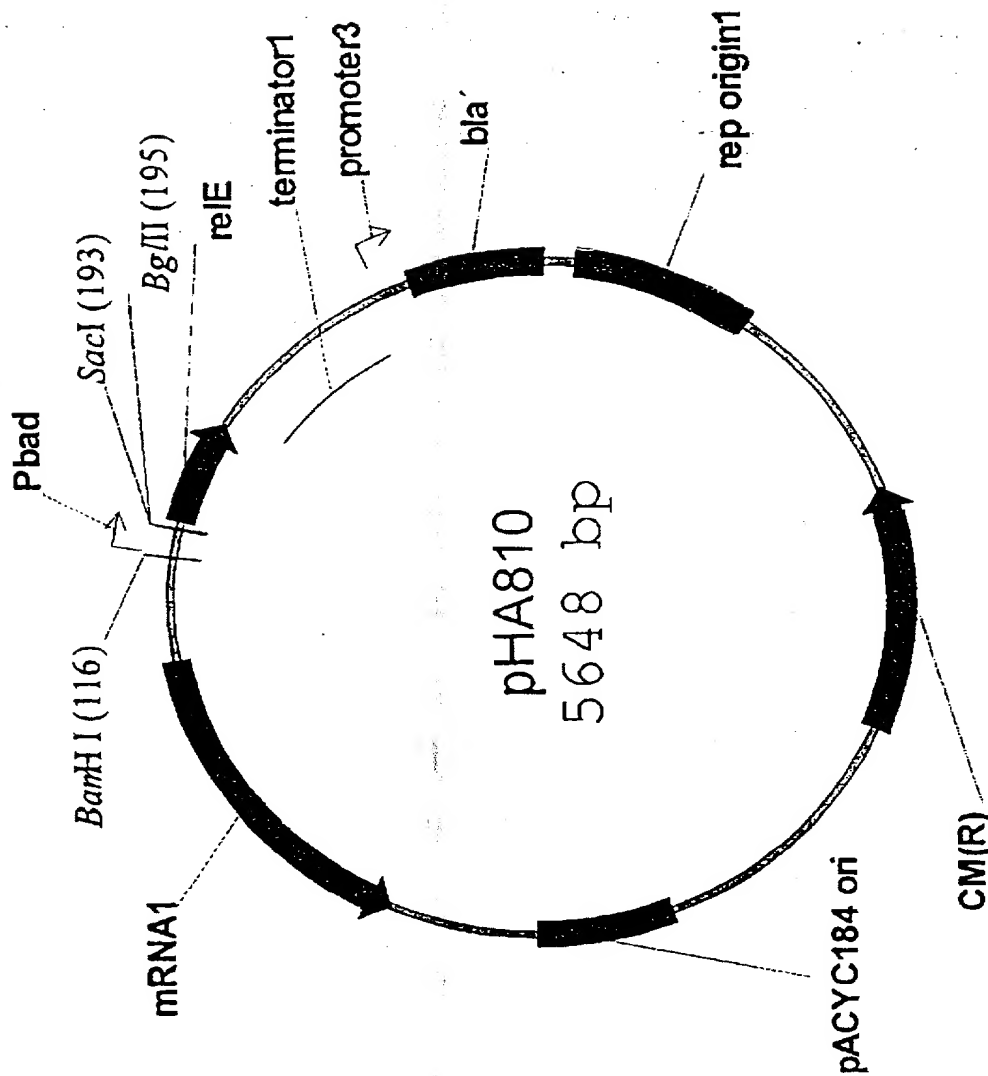


Fig. 18

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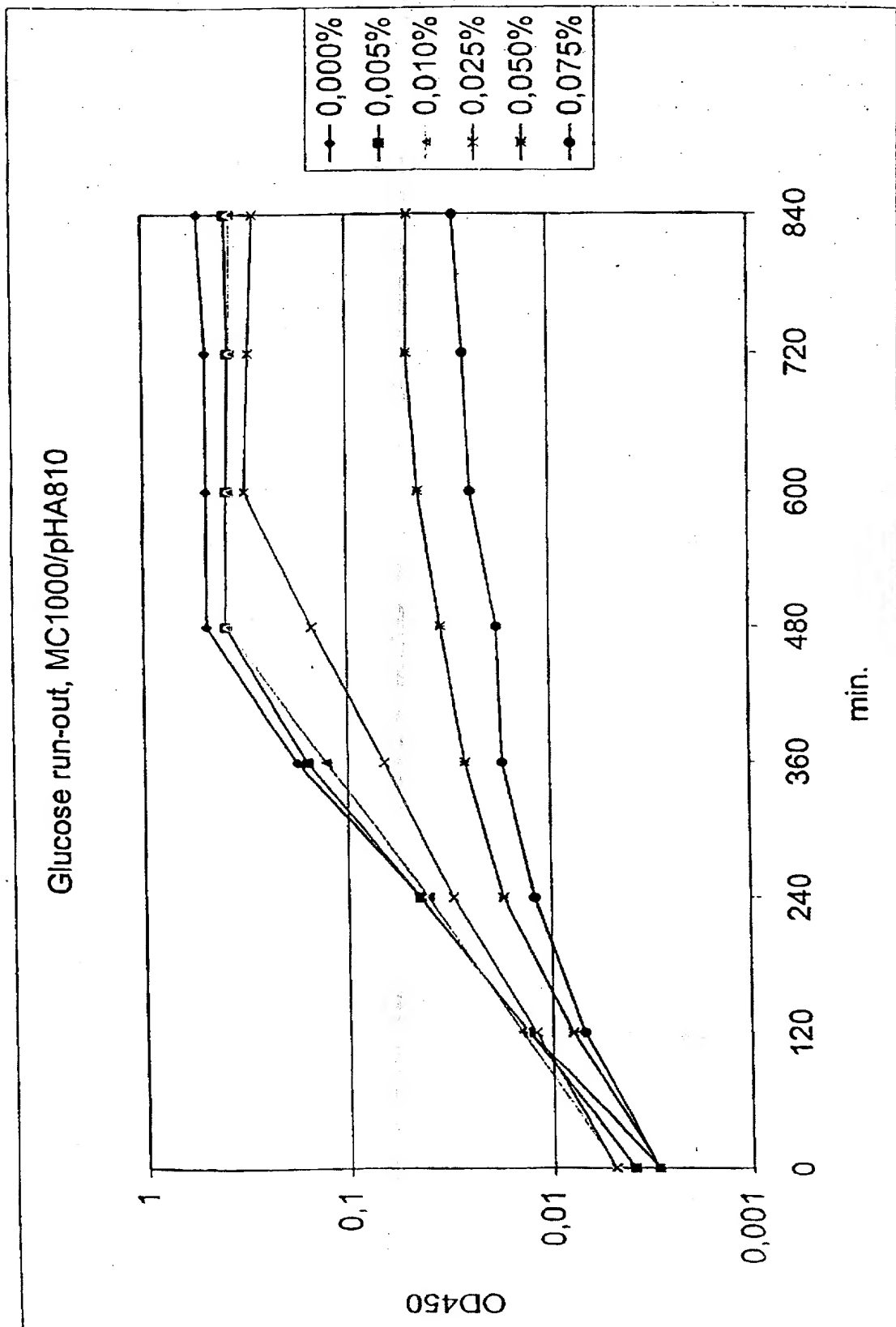


Fig. 19

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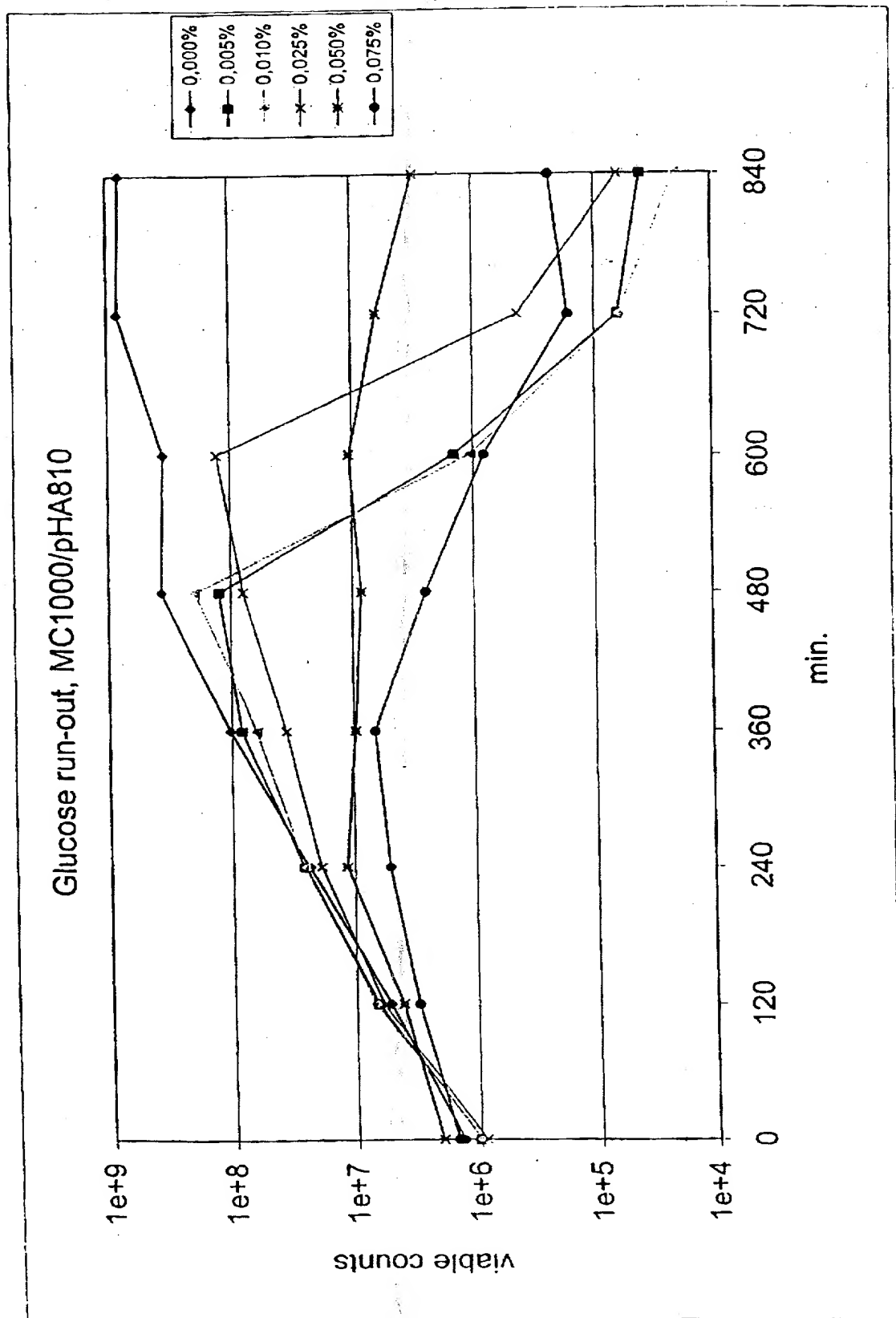


Fig. 20

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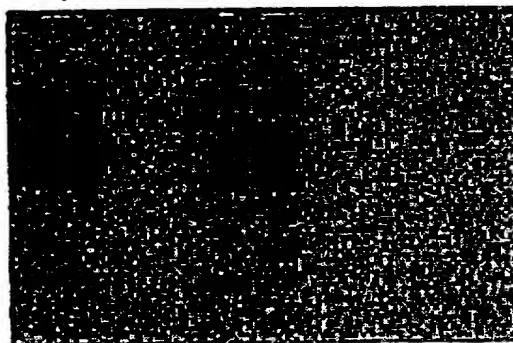


Fig. 21

SUBSTITUTE SHEET (RULE 26)

FIG. 21 DE FIG. 20

DECLARATION - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am an original, first and joint inventor of the subject matter that is claimed and for which a patent is sought on the invention entitled CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT; the specification of which was internationally filed on May 7, 1999, as International Application No. PCT/DK99/00258, and for which the initial documents for entry into the U.S. National Phase were filed on November 7, 2000, and assigned U.S. Serial No. 09/700,130.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56;

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)**Priority
Claimed**

No.: 0627/98	Country: Denmark	Date Filed: May 7, 1998	Yes
No.: 60/085,067	Country: United States	Date Filed: May 12, 1998	Yes

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor: Kenn Gerdes

Inventor's signature Kenn Gerdes

Date 25 September 2001

Residence: Majsmarken 17, DK-5260 Odense S, DENMARK

Citizenship: Danish

Post Office Address: Same as Above

20 Full name of second inventor: Marie Mikkelsen

Inventor's signature Marie Mikkelsen

Date September 25, 2001

Residence: Ørstedsgade 44, 2. th, DK-5000 Odense C, DENMARK

DKX

Citizenship: Danish

Post Office Address: Same as Above

300 Full name of third inventor: Hugo Grønlund

Inventor's signature Hugo Grønlund

Date September 25, 2001

Residence: Uffesvej 26, DK-5200 Odense V, DENMARK

DKX

Citizenship: Danish

Post Office Address: Same as Above

20 Full name of fourth inventor: Kim Pedersen

Inventor's signature Kim Pedersen

Date September 25, 2001

Residence: Solfaldsvej 18-11, DK-5000 Odense C, DENMARK

DKX

Citizenship: Danish

Post Office Address: Same as Above

Full name of fifth inventor: **Peter Kristoffersen**

Inventor's signature *Peter Kristoffersen*

Date September 25, 2001

Residence: **Bispeengen 15, 1. tv, DK-2000 Frederiksberg, DENMARK** *OKX*

Citizenship: **Danish**

Post Office Address: **Same as Above**

Send Correspondence To:
KNOBBE, MARTENS, OLSON & BEAR, LLP
Customer No. 20,995

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Gerdes et al.)
App. No.	:	09/700,130)
Filed	:	Herewith)
For	:	CYTOTOXIN-BASED BIOLOGICAL CONTAINMENT)
Examiner	:	Unknown)

ESTABLISHMENT OF RIGHT OF ASSIGNEE TO TAKE ACTION
AND
REVOCATION AND POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

The undersigned is empowered to act on behalf of the assignee below (the "Assignee"). A true copy of the original Assignment of the above-captioned application from the inventor(s) to the Assignee is attached hereto. This Assignment represents the entire chain of title of this invention from the Inventor(s) to the Assignee.

I declare that all statements made herein are true, and that all statements made upon information and belief are believed to be true, and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that willful, false statements may jeopardize the validity of the application, or any patent issuing thereon.

The undersigned hereby revokes any previous powers of attorney in the subject application, and hereby appoints the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, Customer No. 20,995, as its attorneys with full power of substitution and

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App. No. : 09/700,130
Filed : November 7, 2000

revocation to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected herewith. This appointment is to be to the exclusion of the inventor(s) and his attorney(s) in accordance with the provisions of 37 C.F.R. § 3.71.

Please use Customer No. 20,995 for all communications.

UNIVERSITE LIBRE DE BRUXELLES

Dated: 25 September 2001

By: 

Title:

 **Le Recteur**
Pierre de MARET

 **Le Recteur**
Pierre de MARET 

Address: Avenue F.D. Roosevelt 50
B-1050 Brussels
BELGIUM

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011101

FOBIET DE F00260

Application No.: 09/700,130
Filing Date: Herewith

PATENT
Client Code: PLOUG1,001APC
Page 1

ASSIGNMENT

WHEREAS, We, Kenn Gerdes, a Danish citizen, residing at Majsmarken 17, DK-5260 Odense S, DENMARK; Marie Mikkelsen, a Danish citizen, residing at Ørstedsgade 44, 2. th, DK-5000 Odense C, DENMARK; Hugo Grønland, a Danish citizen, residing at Uffesvej 26, DK-5200 Odense V, DENMARK; Kim Pedersen, a Danish citizen, residing at Søfaldsvej 18-11, DK-5000 Odense C, DENMARK; and Peter Kirstoffersen, a Danish citizen, residing at Bispeengen 15, 1. tv, DK-2000 Frederiksberg, DENMARK, have invented certain new and useful improvements in a CYTOTOXIN-BASED UPON BIOLOGICAL CONTAINMENT for which was internationally filed on May 7, 1999, as International Application No. PCT/DK99/00258, and for which the initial documents for entry into the U.S. National Phase were filed on November 7, 2000, and assigned U.S. Serial No. 09/700,130.

AND WHEREAS, UNIVERSITÉ LIBRE DE BRUXELLES (hereinafter "ASSIGNEE"), with its principal place of business at Avenue F.D. Roosevelt 50, B-1050 Brussels, BELGIUM, desires to acquire the entire right, title, and interest in and to the said improvements and the said Application:

NOW, THEREFORE, in consideration of the sum of One Dollar (\$1.00) to me in hand paid, and other good and valuable consideration, the receipt of which is hereby acknowledged, we, the said inventors, do hereby acknowledge that we have sold, assigned, transferred and set over, and by these presents do hereby sell, assign, transfer and set over, unto the said ASSIGNEE, its successors, legal representatives and assigns, the entire right, title, and interest throughout the world in, to and under the said improvements, and the said application and all divisions, renewals and continuations thereof, and all Letters Patent of the United States which may be granted thereon and all reissues and extensions thereof, and all rights of priority under International Conventions and applications for Letters Patent which may hereafter be filed for said improvements in any country or countries foreign to the United States, and all Letters Patent which may be granted for said improvements in any country or countries foreign to the United States and all extensions, renewals and reissues thereof; and we hereby authorize and request the Commissioner of Patents of the United States, and any Official of any country or countries foreign to the United States, whose duty it is to issue patents on applications as aforesaid, to issue all Letters Patent for said improvements to the said ASSIGNEE, its successors, legal representatives and assigns, in accordance with the terms of this instrument.

AND WE HEREBY covenant and agree that we will communicate to the said ASSIGNEE, its successors, legal representatives and assigns, any facts known to us respecting said improvements, and testify in any legal proceeding, sign all lawful papers, execute all divisional, continuing and reissue applications, make all rightful oaths and generally do everything possible to aid the said ASSIGNEE, its successors, legal representatives and assigns, to obtain and enforce proper patent protection for said improvements in all countries.

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 25 day of September, 2001


Kenn Gerdes


Witness

Date

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 25 day of September, 2001

Maria Mikkelsen
Maria Mikkelsen

[Signature]
Witness

Date

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 25 day of September, 2001

Hugo Grønkind
Hugo Grønkind

[Signature]
Witness

Date

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 25 day of September, 2001

Kim Pedersen
Kim Pedersen

MARTIN TANTOS-ARANDA
Witness

Date

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 25 day of September, 2001

Peter Kristoffersen
Peter Kristoffersen

[Signature]
Witness

Date